DRAFT DETAILED REVIEW PAPER

ON

IN UTERO/LACTATIONAL PROTOCOL

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GARY E. TIMM
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, DC

BATTELLE
505 King Avenue
Columbus, OH 43201

AUTHORS

Rochelle W. Tyl, Ph.D., DABT
Julia D. George, Ph.D.

Research Triangle Institute
Research Triangle Park, North Carolina

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1.0 EXECUTIVE SUMMARY

The purpose of this report is to provide a detailed review paper (DRP) to survey and investigate the status of various protocols that are currently in use, or that have been proposed for use in identifying chemicals that act as endocrine disruptors after *in utero*/lactational exposure. Based on this information, the EPA plans to design an *in utero*/lactational exposure screening study protocol for use in identifying potential endocrine disrupting chemicals.

The United States Environmental Protection Agency (U.S. EPA) is implementing an Endocrine Disruptor Screening Program (EDSP). In 1996, the Food Quality Protection Act and the Safe Drinking Water Act amendments were enacted by Congress to authorize the EPA to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. A two-tiered approach will be utilized. A combination of *in vivo* and *in vitro* screens will be utilized in Tier 1, and Tier 2 will involve *in vivo* testing methods using two-generation reproductive studies. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) will provide advice and counsel on the validation assays.

An *in utero*/lactational exposure assay is proposed as one of the Tier 1 Screening Battery alternate assays. A detailed literature review on the evaluation of endocrine-influenced endpoints after exposure to endocrine disruptors was performed and encompassed: (1) searching the literature databases, (2) contacting individuals to obtain information on unpublished research, and (3) an evaluation of the literature and personal communications. Three candidate protocols were evaluated for their appropriateness for inclusion in the Tier 1 Screening Battery.

The reproductive system is under complex integrative control by the CNS, pituitary, gonads, and the genital tract, all of which are potential targets of transplacental chemical exposure. Postnatal reproductive development can also be altered by changes in synthesis, metabolism, and control of gonadal hormones. In the postnatal period, these changes can be manifested as alterations in sexual differentiation and sexual maturation.

Since effects associated with endocrine disruption may be latent or may not appear until maturation of the reproductive systems, Tier 1 screening must include an evaluation of the endocrine-disrupting activity of the test compound on the postnatal development and maturation of the mammalian reproductive tract. This Tier 1 screen will necessarily encompass critical life stages and processes associated with the endocrine system. Since the developing organism is exquisitely sensitive to endocrine effects, any mammalian assay or test must include exposure to the test compound *in utero* and during lactation, in order to fully evaluate effects on subsequent growth and development. Perinatal exposure provides exposure to sensitive life stages for

reproductive (and other, including CNS) systems, evaluations of offspring beyond weaning to at least puberty, and allows evaluation to detect endocrine-sensitive endpoints in rodents. Gonadal function, estrous cycles, mating behavior, fertilization, implantation, pregnancy, parturition, lactation, weaning, and the offsprings' ability to thrive are all useful endpoints for this type of study. In addition, neonatal survival, growth, and development should also be evaluated. Hormonally induced effects such as abortion, resorption, dystocia, or premature delivery, in addition to physically observable abnormalities, such as masculinization of the female offspring or feminization of the male offspring, can be detected.

During the development of this DRP, a thorough review of the published and unpublished literature was conducted. Over 750 journal articles potentially germane to the effort were reviewed. A Reference Manager Database (RMD) was created from the retrieved literature. The title and abstracts were included in the RMD, along with key information obtained from individual articles such as test chemical and species. In addition, personal interviews with four leading experts in the field of developmental endocrinology were conducted to gather additional information on known testing protocols and endpoints that could be used for identifying impacts from chemicals that can directly interfere with endocrine-driven development.

Three candidate protocols, the FDA Segment III Perinatal Exposure study design, and two proposed designs, one proposed by Drs. L. E. Gray and R. W. Tyl, and one proposed by Dr. R. Kavlock et al., were evaluated with respect to overall study design, appropriateness of endpoints observed and data collected, relevance of data to the detection of endocrine disruption, sensitivity, and statistical power. Based on the information gathered, the study design proposed by Drs. Gray and Tyl was chosen as the most appropriate for evaluation in the prevalidation phase for alternate Tier 1 study designs.

2.0 INTRODUCTION

2.1 DEVELOPING AND IMPLEMENTING THE ENDOCRINE DISRUPTOR SCREENING PROGRAM (EDSP)

In 1996, the passage of the two laws, the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA), authorized the U.S. EPA to screen chemicals found in drinking water sources or food to determine whether they possess estrogenic or other endocrine activity (Federal Register, 2001). Pursuant to this goal, the U.S. EPA is required to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen or other such endocrine effect..." (Federal Register, 2001). The U.S. EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to provide recommendations regarding a strategy for developing a testing paradigm for compounds that may have activities similar to naturally-occurring hormones. Following the recommendations made by EDSTAC in its final report (EDSTAC, 1998), the U.S. EPA established the Endocrine Disruptor Screening Program (EDSP). The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2)

for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. To date, the U.S. EPA has implemented the program on two fronts: (1) the development of the Endocrine Disruptor Priority Setting Database and the approach that will be used to establish priorities for screening compounds, and (2) prevalidation and validation studies on some of the Tier1 assays and Tier 2 tests that are likely to be included in the testing battery. The Endocrine Disruptor Methods Validation Subcommittee (EDMVS) has been set up to advise and review new and ongoing work in the validation of these assays.

2.2 THE VALIDATION PROCESS

The Agency's EDSP is outlined in the December 28, 1998, Federal Register notice (63 FR 71542) and entails *in vitro* methods, *in vivo* screening assays, and reproduction and development toxicity tests. The different screens and tests vary in their scientific development, history of use, degree of standardization, and overall readiness for validation (U.S. EPA, 1998).

The U.S. EPA and EDMVS have chosen to follow the validation process established by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), of which the U.S. EPA was a charter member, for validation of the EDSP screening and testing methods. ICCVAM was established by the National Institute of Environmental Health Sciences as a standing interagency committee to aid in the validation, acceptance, and harmonization of test methods designed to reduce animals use, refine procedures involving animals to make them less stressful, and to replace animal tests wherever appropriate (Federal Register, 2001). To this end, ICCVAM defined a flexible, adaptable framework for test method validation that was applicable to conventional and alternate methods, and could be applied to the needs of different agencies and regulatory processes.

The purpose of validation is to establish the reliability and relevance of a test method with respect to a specific use. The process is science-driven and addresses the scientific principles of objectivity and experimental design (NIEHS, 1997). In addition, as stated in the ICCVAM report, "A test is considered validated when its performance characteristics, advantages, and limitations have been adequately determined for a specific purpose." (NIEHS, 1997). The framework for the validation process outlined by ICCVAM (NIEHS, 1997) is shown in Table 1. It consists of several discrete phases: (1) initial protocol development, (2) prevalidation studies, (3) validation studies, and (4) external scientific peer review. The initial protocol, developed from existing information and experience (past and current research), serves as the starting point for initiating the validation process. Prevalidation studies consist of further development and optimization of specific initial protocols through targeted investigations. Either before or during prevalidation, a detailed review paper addressing all critical areas outlined in *Validation and Regulatory Acceptance of Toxicological Test Methods* (NIEHS 1997) is prepared for each method to summarize, explain, and document decisions regarding the relevant principles, methods, and techniques recommended for the initial protocol. Targeted prevalidation investigations are designed to address questions necessary for completing an optimized, transferable protocol suitable for interlaboratory validation studies. Validation studies consist of comparative interlaboratory studies to establish the reliability and relevance of the protocols developed in the prevalidation stage. Validation requires the development of a detailed review paper to document what is known about the assay system proposed for

validation.

Table 1. ICCVAM Test Method Validation Process

- **Test Development**

- **Prevalidation/Test Optimization**

- Preliminary planning
- Define basis and purpose of test
2. Develop protocol
3. Develop control values
4. Develop data/outcome prediction model

B. Activities
1. Qualify and train laboratories
- Measure intra- and interlaboratory reproducibility
3. Identify limitations of test

- **Determine Readiness for Validation**

- Analyze test development and prevalidation data

B. Standardize protocol

- **Test Validation**

A. Form steering committee/management team
- Define purpose of validation study
- Design study
- Select participating laboratories
- Establish management evaluation and oversight procedures

- Pretest procedures
- Implement data record keeping procedures
- Select reference chemicals
- Code and distribute reference chemicals

C. Test coded chemicals
- Measure interlaboratory performance
- Compile and evaluate data

D. Evaluate test
- Analyze and summarize test results
- Challenge data with prediction model
- Peer review of protocol and data
- Accept, revise, or reject model

V. Submission of Test for Regulatory Approval

A. Prepare report

B. Make supporting data available

C. Prepare results for publication

A test is considered validated when its performance characteristics, advantages, and limitations have been adequately determined for a specific purpose. The measurement of a test's reliability and relevance are independent stages in the validation of a test method, and both are required. Reliability is an objective measure of a method's intra- and interlaboratory reproducibility. If the test is not sufficiently reliable, it cannot be used for its intended purpose. Alternatively, if the test is not relevant, or of questionable relevance to the biological effect of interest, or if it is not an appropriate measure of the effect, its reliability is academic. The relevance of a test may be linked to the mechanism of the toxic effect it measures and to its proposed uses (NIEHS, 1997). The studies conducted will be used to develop, standardize, and validate methods, prepare appropriate documents for peer review of the methods, and develop

technical guidance and test guidelines in support of EDSP. Following the validation studies, results of an external scientific peer review of the study and the optimized protocols will be used to develop the EPA test guidelines.

2.3 PURPOSE OF THE DRP

The purpose of Work Assignment 2-8 is to prepare a DRP to survey and investigate the status of reproductive toxicity studies involving putative endocrine disrupting chemicals, using *in utero*/lactational exposure, and to recommend the next step in developing and validating an appropriate Tier 1 screening study protocol. The preparation of the DRP fulfills objective II.A.1 of the validation process (above), i.e., to define the basis and purpose of the proposed test.

2.4 OBJECTIVE OF THE *IN UTERO*/LACTATIONAL PROTOCOL WITHIN THE EDSP

The EDSTAC (EDSTAC, 1998, Vol. 1, Chapter 5) believed, to the best of its knowledge, that the recommended Tier 1 screening battery, if validated, would have the necessary breadth and depth to detect any currently known disruptors of estrogen, androgen, and thyroid (EAT) hormones. There was concern, however, that chemical substances or mixtures could produce effects from prenatal/prehatch exposure that would not be detected from pubertal or adult exposure. Furthermore, there were differing views with the EDSTAC about whether there is scientific evidence of known endocrine disruptors or reproductive toxicants that can affect the prenatal stage of development without affecting the adult or prematuration stages, and whether effective doses and affected endpoints may differ among the three life stages.

The EDSTAC therefore recommended that EPA take affirmative steps, in collaboration with industry and other interested parties, to attempt to develop a protocol for a full life cycle (i.e., with embryonic exposure and evaluation of the adult offspring) developmental exposure screening assay that can be subjected to validation and standardization. The EDSTAC believed such an assay must involve prenatal or prehatch exposure and retention of offspring through puberty to adulthood and provide structural, functional, and reproductive assessment.

The EDSTAC recognized the difficulty in developing a developmental exposure screening assay that meets both the criteria specified above and the more general criteria for selecting T1S assays set forth in Chapter Three of the EDSTAC report. However, the EDSTAC believed it is worth the effort. Furthermore, the EDSTAC provided brief protocols for *in utero* and *in ovo* developmental screening assays that could be further evaluated for this purpose (an expanded discussion of an *in utero* protocol, similar to this EDSP assay, was included in EDSTAC report, 1998, Volume II, Appendix O).

Finally, the EDSTAC recommended that if such an assay were identified, validated, and standardized, the decision on whether it should be included in the T1S battery should include an evaluation of its potential to replace one or more of the recommended T1S assays and its overall impact to the cost effectiveness of the T1S battery (EDSTAC, 1998, Vol. 1, Chapter 5). The proposed protocol has been identified by the EPA as the "*In Utero*/Lactational Exposure Testing Protocol" and has been assigned for development under the EDSP. RTI, as the lead laboratory for this assay for the EDSP, is suggesting that if this protocol is implemented, it should be used

as a "Tier 1.5" assay or preferably in place of the *in vitro* steroidogenesis and placental aromatase assays, and the *in vivo* male Hershberger assay, the uterotrophic female assay, and either or both pubertal assays.

2.5 METHODOLOGY USED IN THIS ANALYSIS

In order to fully evaluate the appropriateness of the proposed study designs for the EDSP *In Utero*/Lactational Exposure Protocol, a literature search of published articles was conducted. In addition, individuals in the field of endocrine and reproductive studies were contacted to obtain information on unpublished research.

Literature databases accessible through the RTI Information Technology Services were searched for published articles. The literature search was performed in such a way as to include all applicable English language articles and foreign language articles with English language abstracts. English language articles comprised >99% of the articles identified by the search.

Articles were identified through the use of keywords. Keywords were selected to identify research that addressed not only the general scientific areas, but also specific endpoints being considered for inclusion in the EDSP *In Utero*/Lactational Exposure Protocol. Keywords and phrases used, and a more detailed description of the literature acquisition process are presented in Appendix A.

The same methods, sources, and approaches were used to identify and acquire unpublished material and to identify and interview principal investigators in the field. Appendix B contains detailed information concerning this process, including the criteria used for selection of principal investigators, the list of the principal investigators interviewed, the interview questions used, and a summary of the interviews.

2.6 DEFINITIONS

Definitions of terms used in the DRP are presented in Table 2.

Table 2. Terms Used in the DRP

anogenital distance	distance between the genital papilla and the anus
nipple retention	presence of observable nipples on the ventrum of perinatal male rodents
areolae	roughly circular areas of no hair on the ventrum of female fur-bearing mammals in two parallel, longitudinal rows ("milklines") in which are located the nipples of the mammary glands (historical control incidence of areolae in preweaning males is approximately 0-3.5%; females typically exhibit 8-12 nipples plus areolae)
vaginal patency	external opening of the vaginal canal
preputial separation	separation of the foreskin of the penis from the glans
uterotrophic	causing development (maturation) of the uterus
lactational exposure	exposure of offspring through nursing to a chemical administered to the maternal animal during lactation
thyroid development	maturation and differentiation of the thyroid gland
reproductive tract development	maturation and differentiation of the male and female reproductive organs
Mdllerian ducts	embryological structures that mature into the oviduct and Fallopian tubes
Wolffian ducts	embryological structures that mature into the epididymides, seminal vesicles, and vas deferens
male reproductive and accessory organs	all organs that make up the male reproductive system, including testes, epididymides, prostate, seminal vesicles, coagulating glands, preputial glands, bulbourethral (Cowper's) glands, levator ani/bulbocavernosus (LABC) complex, penis, scrotum
rete testis	tubular structures which carry sperm from the seminiferous tubules of the testes to the efferent ducts to the epididymides
female reproductive organs	ovaries, Fallopian tubes (oviducts), uterus, cervix, vagina
multigeneration	evaluation of more than one generation in a testing protocol
gestational day (gd)	specific day during the gestational period
CNS	central nervous system
EAT	estrogen, androgen, and thyroid hormones
postnatal day (pnd)	specific day after birth and before weaning

3.0 SCIENTIFIC BASIS OF THE *IN UTERO*/LACTATIONAL PROTOCOL

3.1 BACKGROUND

Diffusible regulators which control growth, differentiation, development, and homoeostasis can be classified into three systems, depending on the location of the target (cell, molecule, etc.) and the route taken by the regulator, as follows:

- Autocrine System: Molecules made in one location within a cell which affect another molecule within the same cell; the route of dissemination is by intracellular diffusion or other transport process.

- Paracrine System: Molecules made in one cell affect components of another cell located nearby (e.g., between tissues within the same organ, between adjacent organs, etc.); the route of dissemination is by intercellular diffusion or other localized transport.

- Endocrine System: Molecules made in one cell affect components of other cells, tissues, and organs. There can be multiple targets at varying distances from the source. The route of dissemination is via the circulatory system, either free in the plasma or attached to carriers (e.g., steroid hormone binding globulin [SHBG] for steroid hormones).

The endocrine system is composed of glands located throughout the body, hormones which are synthesized and secreted by the glands into the bloodstream, and receptors in the various target organs and tissues which recognize and respond to the hormones. The function of the system is to regulate a wide range of biological processes, including: control of blood sugar (through the hormone insulin from the pancreas), growth and function of reproductive systems (through the hormones testosterone and estrogen and related components from the testes and ovaries), regulation of metabolism (through the hormones cortisol form the adrenal glands and thyroxin from the thyroid gland), and development (cell division, differentiation, morphogenesis, etc.) of an organism from conception through adulthood and senescence. Normal functioning of the endocrine system therefore contributes to homeostasis (the body's ability to maintain itself in the presence of external and internal changes) and to the body's ability to control and regulate development, reproduction, and/or behavior. An endocrine system is found in nearly all animals, including mammals, nonmammalian vertebrates (e.g., fish, amphibians, reptiles, and birds), and invertebrates (e.g., snails, lobsters, insects, and other species). In humans, the system is comprised of more than 50 different hormones, and the complexity in other species would appear to be comparable.

There are four chemicals classes of hormones: (1) steroids derived from cholesterol (e.g., the sex hormones estrogen and androgen), (2) amines synthesized from amino acids (e.g., tyrosine and histidine) giving rise to thyroid hormones and catecholamines (e.g., adrenalin and nonadrenalin), (3) peptides and proteins consisting of chains of amino acids (e.g., growth hormone), and (4) eicosanoids which are derived from a 20-carbon fatty acid called arachidonic acid (e.g., prostaglandins and leukotrienes).

There are also three major classes of receptors to which hormones might bind: (1) receptors found on the surface of cells (to which the peptide hormones bind), (2) receptors found in the cytoplasm of cells (to which the steroid hormones bind), and (3) receptors found in the nuclei of cells (to which the thyroid hormones bind). There are two major mechanisms of hormone action: (1) activation of plasma membrane receptors either via binding or catecholamines, peptides, or protein hormones; and (2) activation of intracellular receptors. Steroid hormones and thyroid hormones operate in this latter manner.

A vast array of receptor proteins and genes are associated with the cells of the body. Cells may contain as many as 10,000 protein receptors for a single steroid hormone, and as many as 50 to 100 genes in a cell may be controlled by the binding of a single type of hormone to the various receptors in a cell. Also, some genes are affected by more than one receptor-hormone complex (EDSTAC, 1998, Vol. 1, Chapter 2).

The focus of the EDSP is to develop, standardize, and/or validate screens to identify disruptors of estrogen, androgen, and thyroid hormone function(s) by one or more of the following mechanisms of action:

- synthesis;
- release into the bloodstream;
- transport and serum binding;
- cell receptors (at cell surface to allow entry into cell);
- nuclear receptors (receptor binding);
- signal transduction (which causes activation of a gene);
- transcription (to generate messenger RNA);
- translation (to generate proteins, e.g., enzymes, regulatory proteins, structural proteins, other receptors, etc.); and/or
- metabolism (in general, to form more polar metabolites by oxidation for urinary excretion, conjugation, activation/inactivation, etc.).

The biochemical events of steroid (estrogen/androgen) hormone action are as follows:

1. Estrogens and androgens (EA) are synthesized in the gonad (i.e., ovary or testis). The adrenal gland also synthesizes androgens for export (and the male brain synthesizes E from A to be used locally; see below).

2. EA are secreted into the blood and transported in free form or bound to a transport protein (i.e.,SHBG). (As an aside, after EA binds to the SHBG, it cannot diffuse into the cell until it is released).

3. Free EA diffuses passively, through the cell membrane, into the cytoplasm of the target cell.

4. Then, EA moves through the nuclear membrane into the nucleus which contains the genetic machinery and the EA receptors.

5. A hormones bind to their receptor (R).

6. Two receptors, each bound to an EA hormone molecule, bind to one another forming a dimer. The receptor dimer binds to a protein transcription factor (TF). This entire complex then binds to a hormone response element (HRE) on a specific gene(s).

7. The gene is subsequently activated, such that the DNA encoding functional information (i.e., an exon) is transcribed by directed synthesis into messenger ribonucleic acid (mRNA).

8. mRNA is transported out of the nucleus into the cytoplasm.

9. The mRNA is "translated" by the ribosomes and additional translational machinery into a protein by linking together the amino acids specified by the mRNA code (which reflects the DNA or gene code).

10. The protein produced can be one of the following kinds of proteins, including, but not limited to, an enzyme, peptide hormone, hormone receptor, growth factor, or structural component.

Each of the steps listed above offers an opportunity for a substance to alter the way hormones exert control over the essential processes in an animal. There are also feedback systems in the body which control the actions of the hormones, increasing hormone production when the amount in the body is too low, and decreasing production when the amount is too large (Figure 1). While in adult organisms, these control mechanisms may help to blunt mild to moderate fluctuations in hormone or hormone-like actions or stresses produced from the environment, feedback systems are less well developed in developing organisms, making these organisms potentially more vulnerable. In addition, it is worth noting that changes in the endocrine system may take place at any point in time during the conception, development, birth, growth, and eventual senescence of the organism or its parents. Such changes may appear as effects in the individual organism and/or in the population (EDSTAC, 1998, Vol. 1, Chapter 2). A brief review of sexual development is presented below. Excellent reviews of pubertal development have also been published by Stoker et al. (2000a) and Goldman et al. (2000).

1

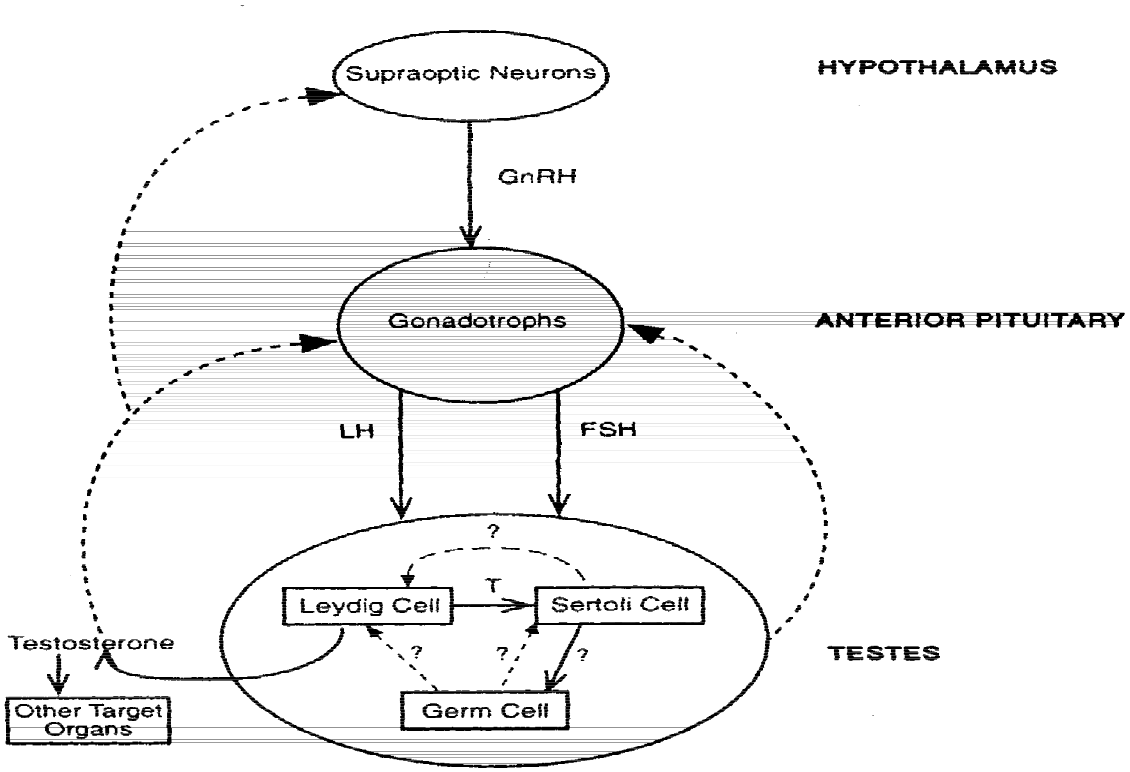


Figure 1. Simplified Overview of the Hormonal Feedback Systems in a Generic Mammalian Male System

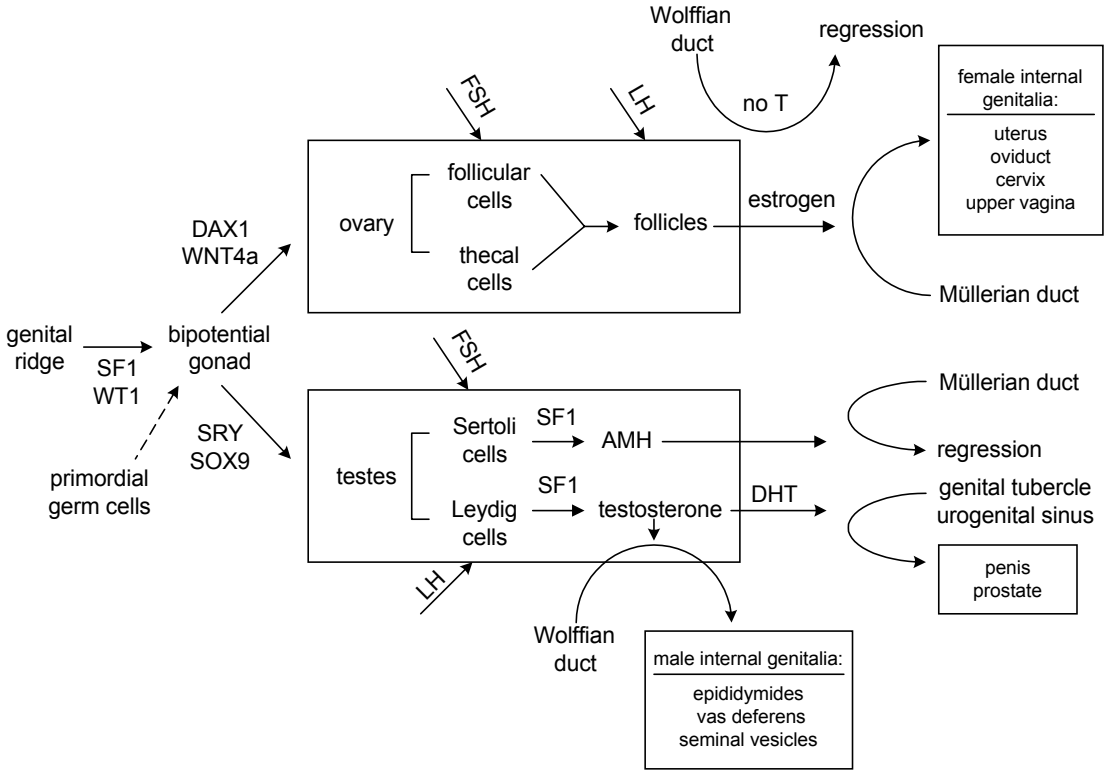
3.2 SEXUAL DEVELOPMENT IN MAMMALS

Primary sex determination concerns the determination of the gonads. In mammals, this determination is strictly chromosomal (established at fertilization). A gene (coding for a DNA-binding protein/transcription factor) on the short arm of the Y chromosome provides a testis-determining factor which organizes the initially indifferent gonad into a testis rather than an ovary. Therefore, the mammalian Y chromosome is crucial for sex determination: an XXXXXY person would be a male (with Klinefelter's syndrome), and an XO person (one sex chromosome only) is a female (with Turner's syndrome, with infertility and other problems, but a female nonetheless).

Secondary sex determination concerns the body phenotype outside the gonads, including accessory sex organs, sex-specific size, musculature, and vocal cartilage (the latter termed tertiary sex characteristics). These secondary (and tertiary) sex characteristics are determined by endocrine hormones secreted from the gonads (Gilbert, 1997; Quigley et al., 1995). The sequences for male and female sexual development are described below and presented graphically in Figure 2. Note that in both sexes, the actual primordial germ cells originate external to the embryo in the yolk sac and migrate into the gonads during their differentiation.

3.2.1 Males

Mammalian embryos of both sexes develop identically until approximately six weeks of gestation in humans and until approximately gd 13-14 in rodents. Both sexes are endowed with an initial genital ridge which converts (under the influence of two genes: SF1 and WT1) to bipotential primordial gonadal tissue, plus two sets of internal genital ducts: Wolffian (mesonephric from the second stage kidney anlagen) and Müllerian (paramesonephric)[1] ducts



(modified from Gilbert, 1997, Chapter 20, Figure 20.2)

Figure 2. Primary Sex Determination of Mammalian Gonads

[1]The mesonephric (and paramesonephric) ducts, which play a role in male mammalian reproductive development, are part of the morphogenetic sequence in the development of the mammalian kidney. Early in development (gd 22 in humans, gd 8 in mice), the pronephric duct arises in the head region of the embryo; the ductal cells migrate caudally, and the anterior region of the duct induces the adjacent mesenchyme to form the pronephric kidney tubules. Those form functional kidneys in fish and amphibian larvae. In mammals, the pronephric tubules and the anterior portion of the pronephric duct degenerate. The more caudal portions persist as nephric

and undifferentiated external genitalia. The trigger for primary sex determination is the activation of the testis-determining factor (i.e., the SRY [sex-determining region of the Y]) to produce a peptide of 223 bases. It is a DNA-binding transcription factor (containing a DNA-binding domain and the HMG [high mobility group] box). The SRY gene-product protein (probably in concert with other factors encoded by autosomal or X-chromosomal genes, such as SOX9) turns on a cascade of genes to induce the indifferent gonadal ridge to begin differentiating into a testis. Testicular differentiation is not an androgen-dependent process. However, androgens, mediated by the androgen receptor (located on target cells which recognize the androgen and respond by activating certain genes, etc.), are absolutely necessary to induce male sex differentiation and development of the male phenotype (Quigley et al., 1995; Gilbert, 1997).

After development of the testis (sex determination), the events of male sex differentiation take two paths; one inhibitory and one stimulatory. The principal function of the inhibiting path is to cause regression of the Müllerian ducts and thus to repress the development of female internal genitalia from the duct: oviducts (fallopian tubes), uterus, and upper vagina. This process occurs in humans between six to eight weeks of gestation, mediated by anti-Müllerian hormone (AMH; also known as Müllerian inhibiting substance), a glycoprotein hormone (a member of the transforming growth factor-beta family), secreted by the Sertoli cells in the seminiferous tubules of the testis (Quigley et al., 1995). Testosterone (see below) produced in the fetal testis inhibits the development of the breast primordia (Gilbert, 1997) and causes regression of nipples in perinatal males.

The stimulatory events of male sex differentiation require high levels of androgens and a functional androgen receptor (AR). Androgens are required to stabilize the Wolffian duct system to prevent its involution and to induce differentiation of the Wolffian ducts into the epididymides, vasa deferens, and seminal vesicles. The events of internal duct differentiation (which occur in humans between nine and 13 weeks gestation and in rodents on gd 13-15) are induced by the action of testosterone itself, probably via a paracrine effect, due to its high local concentrations in the vicinity of the Wolffian ducts, which are in close proximity to the testis. Testicular testosterone synthesis and secretion by fetal Leydig cells (located interstitially outside the seminiferous tubules within the testis) begins at eight weeks gestation and peaks at 11-18 weeks gestation in humans (in rodents this process occurs during gd 14-19). Major control of testosterone synthesis during this time is by maternal chorionic gonadotropin from the placenta,

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ducts and become part of the excretory system. As the pronephric tubules degenerate, the mid portion of the nephric duct initiates a new set of kidney tubules in the adjacent mesenchyme. This set constitutes the mesonephric kidney (humans form approximately 30 such tubules around pnd 25). As more tubules are induced caudally, the anterior mesonephric tubules regress. In female mammals, the regression is complete; in male mammals, some of the mesonephric tubules persist to form the vas deferens and efferent ducts (the sperm transporting tubes) of the testis. (The permanent kidney of amniotes, the metanephros, is formed from branching of the paired nephric ducts to form ureteric buds which enter the adjacent metanephrogenic mesenchyme to differentiate into nephrons of the mammalian kidney. The ureteric buds separate from the nephric ducts to become the ureters, which carry urine from the kidney to the urinary bladder.)

although other factors (such as the genes SOX9 and SF1) may also be involved. The potent metabolite of testosterone, dihydrotestosterone (DHT), is not involved in the process of internal masculinization *in utero*, since the enzyme, 5-alpha reductase, required to convert testosterone to DHT, is not expressed in these tissues until about 13 weeks gestation in humans and gd 15-16 in rodents, at which time the process of internal masculinization is complete.

DHT is required for development of the prostate and prostatic urethra from the urogenital sinus and masculinization of the external genital primordia (the genital tubercle, urethral folds, and labioscrotal swellings) into the penis, penile urethra, and scrotum. The enzyme necessary to convert testosterone to DHT is present in these tissues at this time (weeks 9-13 in humans and gd 13-15 in rodents). A functional AR is absolutely required to mediate the actions of testosterone and DHT in inducing the expression of androgen-dependent genes necessary for internal and external genital masculinization (Quigley et al., 1995; Gilbert, 1997).

The hypothalamic-pituitary-gonadal (hpg) axis begins functioning at the start of puberty. The hypothalamus in the brain begins to produce and secrete gonadotropin-releasing hormone (GnRH) which travels via a specialized portal system of the systemic circulatory system directly to the anterior lobe of the pituitary (the hypophysis; just under the brain, attached by a stalk). In a receptor-mediated process, GnRH stimulates the production and secretion of two gonadotropins (substances which affect the gonads). The two are luteinizing hormone (LH) and follicle stimulating hormone (FSH), named for their roles in pregnant female mammals, but necessary for puberty of both sexes. In males, FSH and LH travel via the systemic circulatory system to the testes. In the testes, in receptor-mediated processes, LH stimulates the interstitial cells of Leydig to trigger a burst of testosterone synthesis. Testosterone, in AR-mediated processes, within and outside the testis, as previously described, induces the formation of male secondary and tertiary sex characteristics and the onset of spermatogenesis (DHT does not appear to be involved). There is some evidence that FSH and prolactin (Prl) up regulate the LH receptors in the Leydig cells to increase the response to LH stimulation. FSH stimulates the Sertoli cells (in the seminiferous tubules) to differentiate to become the "nurse cells" to the developing germ cells in the presence of high concentrations of testosterone (EDSTAC, 1998, Vol. 1, Chapter 2).

3.2.2 Females

Primary female sex determination in mammals requires at least one X chromosome (two are the norm) and no Y chromosomes, and the formation of paired ovaries. Secondary sex determination requires the formation of a vagina, cervix, uterus, oviducts (Fallopian tubes), mammary glands and sex-specific size, vocal cartilage, and musculature (tertiary sex characteristics). The secondary sex characteristics are usually determined by the hormone(s) secreted from the gonads. However, female phenotype can be viewed as a "default" alternative. In the absence of *in utero* female sex hormones (or even in the absence of female gonads removed before differentiation), rabbits (whether XX or XY) were female in appearance, with oviducts, uterus, and vagina (and no penis or male sex accessory organs) (Gilbert, 1997).

The bipotential gonad, in the absence of SRY (and in the presence of products from genes DAX1 and WNT4a; Gilbert, 1997, p. 781), follows the female pathway to make ovaries. Cells of the ovaries differentiate into follicular and thecal cells, which together synthesize estrogen and form follicles surrounding the female germ cells. Under estrogen, from the fetal gonads, the Müllerian duct remains intact and differentiates into the oviducts, uterus, cervix, and upper vagina. The Wolffian duct, deprived of testosterone, degenerates. The hpg axis plays a comparable role (to that of the male) in female puberty, whereby pituitary FSH stimulates the granulosa and thecal cells in the follicles of the ovary to trigger a burst of estrogen synthesis, and LH enhances receptivity of the follicles to estrogenic stimulation and triggers the continuation of oogenesis (no primary oocytes are formed postnatally) and the beginning of estrous cyclicity and ovulation.

3.3 SEX-SPECIFIC BEHAVIORS

One aspect of secondary sex determination involves the development of sex-specific behavior and the effects of hormones on the central nervous system. Penile thrusting in rats is a male-specific behavior. It is controlled by motor neurons to the levator ani and bulbocavernosus muscles. These neurons originate from a spinal nucleus which specifically concentrates testosterone. In female rats, these muscles are vestigial, and the volume of the controlling neurons is greatly reduced. In fetal and neonatal rats, testosterone prevents the "normally" occurring cell death (apoptosis) of the neurons in this region. Female rats lose up to 70% of the neurons in this spinal nucleus, while male rats lose only 25%. Castration of an adult male rat causes 50% reduction of the soma (cell body) area and dentrite length of the neurons in this spinal nucleus; the reduction is reversed by injection of testosterone. In the mammalian brain, estrogen-sensitive neurons are also present. These neurons are located in the areas which are known to mediate reproductive behavior: the hypothalamus, pituitary, and amygdala. Estrogen alters the electrical and chemical characteristics of the estrogen-sensitive hypothalamic neurons. The electrical activity varies during the estrous cycle, with maximal activity during ovulation. Estrogen also stimulates neurons in the brain regions which induce female reproductive behavior. Ovariectomized female rats given estrogen injections directly into the hypothalamus exhibit lordosis (a position which stimulates mounting behavior in males); control ovariectomized females do not exhibit lordosis (Gilbert, 1997, p. 786).

One current hypothesis proposes that perinatal exposure to certain hormones imposes permanent, sex-specific changes in the central nervous system, in the brain regions (predominantly in the hypothalamus) which regulates "involuntary" sexual physiology (organization). In contrast, during adult life, exposure to the same hormones may have limited, transitory, activational effects (i.e., the "organization/activation hypothesis") (Gilbert, 1997, p. 787).

Examples of "organization" exposures and their consequences include:

- The cyclic secretion of LH by the adult female rat pituitary is dependent on the lack of testosterone during the first week of life (estrous cyclicity does not normally occur until after puberty at approximately pnd 31). If testosterone is administered to female rats on pnd 4, LH secretion becomes noncyclical in them as adults.
- Removal of male newborn pup testes (major source of testosterone) results in cyclic secretion of LH in them as adults.
- Male brain pattern is determined by estradiol (17β-estradiol is the endogenous and most potent estrogen in mammals) in the perinatal period. Testosterone in fetal or neonatal blood is converted to estradiol by the P450 enzyme aromatase locally in the male brain in the hypothalamus and limbic system, the two brain areas known to regulate reproductive and hormonally-dependent behaviors.
- Estradiol in the female is also converted from testosterone (or androstenedione) locally in the ovary (by aromatase and the interaction between the follicular and the thecal cells of each follicle).
- The fetal environment is rich in estrogens from the maternal gonads and the placenta (placental aromatase in mammals with long-term gestation is also very active to convert testosterone to estrogens). Estrogen is prevented from masculinizing the nervous system of the female fetus by its binding to alpha-fetoprotein (in both sexes) and the absence of the ER in many fetal brain areas in the female. Alpha-fetoprotein (AFP) is made in the fetal liver and released in large quantities into the circulation and the cerebrospinal fluid; it binds estrogens but not testosterone (Gilbert, 1997, p. 787). Therefore, estrogens cannot cross the blood-brain barrier, and the only estrogen in the male brain is that made locally.
- Many mammals, including the rat and human, have a brain nucleus (neuronal cell cluster) termed the "sexually dimorphic nucleus of the preoptic area" (SDN-POA) which is four times larger in normal male rats than in female rats. The development of the SDN-POA occurs during the estrogen-sensitive period of brain sexual differentiation (Liaw et al., 1997). Estrogenic xenobiotics can increase the size of SDN-POA in female fetuses *in utero* (i.e., masculinize them) (see nos. 3 and 5).

Females with XO genotypes (Turner Syndrome: 45 chromosomes, 22 pairs of autosomal chromosomes and one X chromosome; 45, X) develop ovaries, but they atrophy before birth and the germ cells die before puberty. However, under the influence of estrogen derived from the fetal ovary and then from the mother and placenta, these infants are born with internal and external female genitalia (Gilbert, 1997, p. 783).

Males with XY genotype and with the testis-determining factor gene on the Y chromosome, therefore, make testes, and the testes make testosterone and convert it to DHT (with the appropriate enzyme present). However, due to one of several mutations in the androgen receptor (AR), the AR on the target tissues for testosterone and DHT cannot recognize or respond to testosterone or DHT. They can, however, respond to the anti-Müllerian hormone (AMH), so the Müllerian duct degenerates. Because they can respond to estrogen made by the adrenal glands, they are distinctly female in external appearance. They do not have a uterus or oviducts, and they do have mid-abdominal testes which do not descend. This condition is termed androgen insensitivity syndrome (AIS; Quigley et al., 1995; Gilbert, 1997).

An autosomal recessive mutation in the gene for 5-α-reductase (which converts testosterone to DHT, the potent *in utero* regulator of male external genitalia) in humans (in the homozygous state) results in chromosomally male newborns which appear female. At puberty, when a surge of testosterone is initiated, these children develop male external genitalia and secondary and tertiary male sex characteristics. This situation was first discovered in a small South American village (the syndrome in Spanish translates into "twelve year old penis") and provided the first information on the role of DHT in male external sexual development (Imperato-McGinley et al., 1974).

3.4 APPROPRIATENESS OF ENDPOINTS FOR MEASURING THE ENDOCRINE DISRUPTION AFTER *IN UTERO*/LACTATIONAL EXPOSURE

Hazard-based study designs must use endpoints that have been shown to be robust, reproducible, appropriately sensitive, biologically plausible, and relevant to the adverse outcomes of concern. Definitions of the attributes of such endpoints are as follows:

Reproducible: These endpoints must be reliable; the same findings occur under the same conditions within the initial reporting laboratory (intra-laboratory) and among other laboratories (inter-laboratory). If the results from endpoints are not reproducible, they cannot form the basis for future research and are most likely not useful for hazard assessment.

Robust: These endpoints must be present after comparable routes of exposure, (e.g., dosed feed or dosed water). The use of oral gavage, a bolus dose once/day, may result in exacerbation of the endpoint if the parent material is the proximate toxicant and is metabolized to a nontoxic metabolite, if bolus dosing overwhelms the metabolic capacity of the organism or preparation, or it may result in diminution or loss of the endpoint if the parent compound must be metabolized to the active form. Different effects may be observed by non-oral routes, such as inhalation, topical application, injection, etc., since these routes bypass "first-pass" metabolism by the liver. The findings from routes unrelated to human or environmental exposures may not be useful for hazard assessment. These findings must also be present at the same routes and doses over time.

Sensitive: These endpoints should not be dependent on unique conditions (e.g., intrauterine position [IUP], etc.), especially those which are not relevant to the species at risk. These endpoints should not exhibit high variability (insensitive) or be greatly affected by confounders (too sensitive).

Relevant: These endpoints must be biologically plausible and related to adverse effects of interest/concern. If there are no adverse effects at the dose/duration/route evaluated, these endpoints should be predictive of other adverse effects at higher doses, after longer exposure duration, and/or by different routes, etc.

Consistent: These endpoints must occur in the presence of effects in other related, relevant endpoints, if possible, at the same dose, timing, duration, routes of exposure, etc.

Individual candidate endpoints are discussed below.

- **Anogenital distance.** The sex differences in anogenital distance (AGD; measurement of distance from anus to genital papilla) at birth and beyond (male AGD is approximately twice as long as female AGD in rats and mice) are under androgen control, specifically DHT (Gray et al., 1998; Gray and Ostby, 1998), and do not appear to be affected by estrogens (Biegel et al., 1998a) but are affected by pup body weights (Ashby et al., 1997a). Recent data (Gallavan et al., 1998) from 1501 control CD® (SD) rat pups indicated that a 1 gm increase in body weight results in a 0.19 mm increase in AGD. If AGD values are shorter in either sex (especially if in both sexes) in a treatment group with reduced pup body weights, it is likely that the AGD effect is confounded by the body weight effect. Exposure to anti-androgens *in utero* results in shortened AGD in males, with no effects on female AGD (McIntyre et al., 2000). The precision with which laboratories measure AGD on newborns ranges from use of a dissecting microscope with an ocular micrometer and eyepiece grid (and the pup flat on the microscopic platform) to hand-held pups and a ruler. Obviously, the accuracy and variance of the values will differ, depending on the method. Precise methods result in very tight values which may result in statistically significantly different group means. This parameter is very sensitive; the biological significance and relevance of changes in AGD are unknown. AGD has also been shown to be significantly reduced in CD® (SD) newborn rats whose dams were on 50% feed restriction from gd 7 (Carney et al., 1998).

  This is considered to be a very appropriate endpoint. It is DHT-mediated, and the endocrine-mediated effects persist into adulthood. It is also the most sensitive endpoint from *in utero* exposure to an anti-androgen (McIntyre et al., 2001). However, since it is confounded by body weight, the current practice is to present the data as mm, mm/cube root of the body weight, and/or to analyze the data by ANCOVA (analysis of covariance), with the body weight at measurement (birth, weaning, etc.) as the covariate. These procedures help to account for differences in body weight (especially in groups where there is systemic toxicity, expressed as reduced parental and offspring body weights).

- **Retention of nipples in preweanling males.** This is evaluated usually in males on pnd 11-13 and is DHT-mediated. Effects persist into adulthood. In the authors' laboratory, retained nipples have never been observed in control preweanling CD® (SD) males, although areolae have been observed in the author's laboratory in 0-3.5% of control males on pnd 11-13 (based on examination of over 3000 males *in toto*). This is a sensitive indicator of altered testosterone (T) and/or DHT levels (effects on synthesis, degradation, receptor binding, transcriptional activation, etc.). Male pups with retained nipples are more likely to exhibit reproductive system malformations, but the correlation is not perfect (i.e., some males with nipples exhibit no malformations, some males with no nipples do exhibit malformations). Retention of nipples is also a reasonable low-dose predictor of male reproductive malformations caused by perinatal exposures at similar or higher doses. In some instances, permanent nipples may qualify as a malformation, due to relative permanence (McIntyre et al., 2001).

- **Puberty.** Acquisition of puberty can be determined in both females and males by a number of physical changes. For females, vaginal patency and age of first estrus are most often used, whereas in males, preputial separation is most often monitored. In both sexes, acquisition of puberty is affected by body weight, so the current approach is to co-vary the age at acquisition by the body weight at weaning, or by some measure of weight gain during the postlactational, prepubertal period (the selection of the end date for weight gain is problematic). Typically, pubertal delays of one to three days (in both sexes) are almost always accompanied by reduced body weights (e.g., Tyl et al., 1999, 2001a,b). Acceleration in one sex and delay or no effect in the other sex is more likely due to endocrine-mediated effects (e.g., Chapin et al., 1999; Biegel et al., 1998a,b). Since acquisition of puberty involves processes that, in part, become active after birth, exposure to putative endocrine disruptors during the postnatal period is likely to give a more complete indication of the effects of the compound. Extension of exposure into the postnatal period also provides the opportunity to affect endocrine-dependent processes that are maturational and do not result in frank malformations of the reproductive system, since malformations make evaluation of other ongoing processes problematic.

  - **Vaginal patency in females.** In females, acquisition of puberty is indicated by vaginal opening or patency (VP). VP is dependent on 17ß-estradiol. In control CD® (SD) rats in the authors' laboratory, the grand mean age at VP is 31.1 days (based on 15 studies from 1996 to 2000). VP may be observed first as the appearance of a small "pin hole", but is typically recorded as acquired when vaginal opening is complete (with notation if a vaginal thread persists).

  - **Age of first estrus in females.** Within a few days post-VP, the female exhibits her first estrus, so age at first estrus (absolute age and/or interval from VP to first estrus) is also useful. Late follicular growth of the first ovulatory cells is stimulated about the time of vaginal opening, although there is some variation in the initial release of oocytes. Following vaginal opening, daily vaginal smears are monitored to determine the age of first estrus or first vaginal cycle. Irregular

estrous cycles are often seen in the immediate post-pubertal period (Goldman et al., 2000).

— **Preputial separation.** Acquisition of puberty in males is indicated by preputial separation (PPS; balanopreputial separation) or separation of the foreskin of the penis from the glands. PPS is dependent on androgens. PPS is a process that leads to the cleavage of the epithelium through cornification, forming the squamous lining of the prepuce of the penis (Stoker et al., 2000a). As a sign of puberty and an essential prerequisite for further development of the ejaculatory process, PPS has been used as a reliable, noninvasive endpoint by which to monitor rodent pubertal development and perturbations of this process. This landmark of acquisition generally occurs during the peripubertal period (pnd 36-55 or 60; Goldman et al., 2000). In control CD® (SD) rats in the authors' laboratory, the grand mean age at PPS is 41.9 days (based on 15 studies from 1996 to 2000).

— **Testis descent in males.** A less useful landmark of acquisition of puberty in males is testes descent (into the scrotal sacs from the abdominal cavity through the inguinal canal and ring), which occurs during lactation (pnd 15-20) and may be mediated by T and/or DHT.

‘ **Estrous cyclicity in postpubertal females.** After the initial release of ova, female rats begin to exhibit four- to five-day estrous cycles, with accompanying changes in vaginal cytology and circulating hormones. The acquisition of estrous cyclicity results from shifts in the hypothalamic-pituitary-ovarian endocrine axis and is the culmination of the maturation of reproductive processes that began prenatally. As indicated above, irregular estrous cycles are more common in the first weeks after acquisition of VP.

‘ **Organ weights.**

— **Reproductive (including accessory sex organ weights).** Reproductive organ weights should be obtained at adulthood and should include: (a) ovaries and uterus for females, and (b) testes, epididymides, prostate (whole, and dorsolateral and ventral lobes separately; dissection may be postfixation), seminal vesicles, coagulating glands, preputial glands, bulbourethral (Cowper's) glands, and levator ani/bulbocavernosus (LABC) complex for males.

— **Adrenals.** These are important endocrine organs and are a secondary source of steroid sex hormones, specifically T.

— **Thyroid.** Thyroid hormones (T3 and T4) are necessary for normal growth, development, differentiation, and regulation in most organ systems (Goldman et al., 2000; Stoker et al., 2000a). Disruption of the feedback control of thyroid function may result in either a hypertrophic (goiter) or hypotrophic thyroid,

depending on the mechanism of disruption. These changes would be evident in the weight of the thyroid gland.

— **Systemic (liver, kidneys, brain, etc.).** Systemic organ weight should be obtained at adulthood in both sexes and should include liver, adrenal glands, pituitary, brain (regions), etc. Comparison of the effect of the test compound on these organ weights (absolute and relative) to effects on reproductive organ weights will provide a more complete characterization of toxicity, and suggest whether observed toxicity is more or less targeted to the endocrine system.

— **Absolute and relative to body weight (and brain weight).** Organ weights (both reproductive and systemic) should be presented as absolute and relative to terminal body weight. Relative weights will correct for effects on body weights (i.e., systemic toxicity). An alternate index of change (to be used in conjunction with organ weights relative to body weights) is organ weights relative to terminal brain weight. Brain weight is generally more stable than body weight in the presence of exogenous compounds and provides a context for interpreting changes in organ weight. Dr. J. Haseman (NIEHS/NTP, personal communication to Dr. R. W. Tyl, August, 2000) has indicated that organ weight that differ significantly both as absolute and relative (to body or brain weight) and in the same direction (i.e., both increased or both decreased) should be considered biologically relevant. An alteration in organ weights, in the presence of altered body (and/or brain weight) typically exhibits a significant change in absolute weight (usually reduced) and no change of and increased value for relative organ weight. These effects are most likely due to reduced body weight as the primary effect with secondary effects on organ weight.

‘ **Behavioral assessments.** Maternal and neonatal behaviors involving nesting and nursing are under the control of the endocrine system. Qualitative evaluation of these behaviors, as they affect viability and ability to thrive, provide another measure of possible endocrine disrupting activity of a test compound.

‘ **Reproductive development.** Reproductive development involves both morphological and hormonal aspects, which operate together to result in correctly formed and responsive reproductive systems in both males and females. In mammals, gonadal origins begin early in embryonic development, prior to sexual differentiation (Schardein, 1999). Initial stages are the same for both male and female. Sexual differentiation and maturation are under hormonal control. Thus, both physical and hormonal indicators of reproductive development can be monitored to detect the presence of endocrine-disrupting activity.

— **Gonads (male and female development).** Initially, the gonads appear as a pair of indifferent longitudinal genital ridges in the embryo. The primordial germ cells invade the genital ridges at about gd 10-12 in the rat. Concomitantly, the genital ridges form primitive sex cords, which are indistinguishable, male from female. In the male, the primitive sex cords continue to proliferate and form the

testes. The cords extend into the connective tissue, forming a network of internal or medullary sex cords and, at the most distal end, the rete testis. The testicular cords lose contact with the surface epithelium and become separated from it by a thick extracellular matrix, the tunica albuginea. Thus, the germ cells are found in the cords within the testis. At puberty, the cords hollow out to form the seminiferous tubules, and the germ cells begin sperm production. The sperm are transported out of the testis by the rete testis, which joins the efferent ducts (from the mesonephric duct) to the epididymides, which link to the Wolffian duct, which forms the vas deferens. During the fetal period, the cells outside the seminiferous tubules, the interstitial mesenchymal cells, differentiate into the Leydig cells, which synthesize testosterone. The cells of the testis cords (not the germ cells) differentiate into the Sertoli cells, which support, protect, and nurture the developing sperm and secrete the anti-Müllerian duct hormone.

After formation of the primitive sex cords, genetically female embryos undergo differentiation as the primitive sex cords proliferate to form the ovaries. The initial sex cords in females do not continue to proliferate but degenerate. The epithelium then produces a new set of sex cords which stay near the outer surface (cortex) of the organ. They are, therefore, termed cortical sex cords, and the germ cells will reside in this area. These secondary cortical cords split into clusters, and each cluster surrounds a germ cell. The germ cells will become ova, and the surrounding epithelial sex cords will differentiate into granulosa cells. The mesenchymal cells in the cluster will differentiate into thecal cells. Together, the granulosa and thecal cells will form the follicles that envelope the germ cells (one germ cell per follicle) and secrete steroid hormones.

— **Wolffian duct (male development).** The epididymides, vas deferens, ventral prostate, and seminal vesicles are formed from the embryonic structure known as the Wolffian ducts in the male. The Müllerian ducts (characteristic of female development) regress as male sexual differentiation proceeds.

— **Müllerian duct (female development).** In the female, the ducts form the oviducts, uterus, cervix, and upper vagina.

— **Pre- and postnatal development.** Visual examination of the reproductive tracts of both males and females at birth, and during the postnatal period, provides a measure of both pre- and postnatal development as described above and below.

— **Puberty.** Acquisition of puberty, determined by the age (in days) of acquisition of VP in offspring females and the age (in days) of acquisition of balanopreputial separation (PPS) in males, can be used to compare the relative effects of a compound on male and female reproductive development. In the author's laboratory, acquisition of these indicators of puberty is consistent with very tight variances intra- and inter-studies.

Statistically significant differences in age at acquisition of puberty may indicate endocrine-mediated effects, especially if the effects are different for the sexes (e.g., VP is delayed and PPS is accelerated or unchanged, VP is accelerated and PPS is delayed or unchanged, etc.) and if the effects are profound (acceleration or delay of many days versus only a few days). However, acquisition of developmental landmarks is dependent on both age and weight (i.e., heavier animals acquire the landmark earlier, while lighter animals acquire the landmark later), but lighter animals do acquire the landmark (unless there is another cause for the delay) and in many cases acquire the landmark at a lighter weight than the heavier animals. This observation is consistent with the recognition by the U.S. EPA (1996, p. 56295) that "body weight at puberty may provide a means to separate specific delays in puberty from those that are related to general delays in development." The significance (i.e., the consequence, if any) and "the biologic relevance of a change in these measures of a day or two is unknown" (U.S. EPA, 1996, p. 56295).

The suggestion is to obtain body weights on the day of acquisition and to analyze the acquisition data by analysis of covariance (ANCOVA), with the body weight at acquisition as the covariate. There is some discussion that the body weight at acquisition is not the appropriate covariate, but rather the body weight at birth (this is not the author's experience and requires uniquely identifying the pups at birth by tattoo) or the body weight at weaning (pnd 21). The rationale is that the body weights during the process of sexual development, leading to puberty, may be more relevant than the body weights at puberty after almost all the reproductive development has already occurred.

The recognition that body weight is important in analyzing and understanding acquisition of puberty is strengthened by the work of Kennedy and Mitra (1963), who showed that body weight and food intake are factors in the initiation of puberty in the rat, and by the work of Carney et al. (1998), who put groups of 26 timed-mated SD rats on standard diets at 100% (control), 70%, or 50% of historical control feed intake levels from gd 7 through weaning on pnd 21. Selected weanlings were continued on feed restriction until 10 weeks (with 100% feed from 10 to 20 weeks of recovery) or until 20 weeks of age, with necropsy of all offspring at 20 weeks of age. Feed restriction resulted in reduced weight gains for dams and pups relative to the degree of restriction. In both the 50% and 70% of control feed restriction groups, gestation length was significantly increased, and age at VP and PPS was also delayed (by 1 day at 70% restriction and by 6 days at 50% restriction for both parameters). Anogenital distance at birth was significantly reduced in both sexes in the 50% restriction group, but AGD:body weight ratios were essentially identical across groups, indicating that smaller (low body weight) pups had shorter AGDs and that the effects were proportional. The authors conclude that "these results show that certain reproductive and developmental endpoints are altered by feed restriction in the range relevant to common testing scenarios" (Carney et al., 1998). However, Stoker et al. (2000b) showed that a 10% decrease in body weight at weaning did not affect PPS.

- **Hormonal controls.** The endocrine system, also referred to as the hormone system, is made up of glands located throughout the body, hormones that are synthesized and secreted by the glands into the bloodstream, receptors in the various target organs, and tissues that recognize and respond to the hormones. The function of the system is to regulate a wide range of biological processes, including control of blood sugar (through the hormone insulin from the pancreas), growth and function of reproductive systems (through the hormones testosterone and estrogen and related components from the testes and ovaries), regulation of metabolism (through the hormones cortisol from the adrenal glands and thyroxin from the thyroid gland), development of the brain and the rest of the nervous system (estrogen and thyroid hormones), and development of an organism from conception through adulthood and old age. Normal functioning of the endocrine system, therefore, contributes to homeostasis (the body's ability to maintain itself in the presence of external and internal changes) and to the body's ability to control and regulate reproduction, development, and/or behavior. An endocrine system is found in nearly all animals, including mammals, nonmammalian vertebrates (e.g., fish, amphibians, reptiles, and birds), and invertebrates (e.g., snails, lobsters, insects, and other species). In humans, the system comprises more than 50 different hormones, and the complexity in other species appears to be comparable.

  - **Hypothalamus (GnRH).** It is generally believed that the CNS is the trigger point for initiation of sexual maturation in the male and female rat (Goldman et al., 2000; Stoker et al., 2000a). GnRH is present in the fetal brain and slowly increases until the second postnatal week in females, and the third postnatal week in males. At that point, GnRH increases steeply and remains elevated until puberty. At puberty, the GnRH neurons undergo a morphological change, developing spiny-like processes that may be related to an increase in synapses on the cells. It has been shown that at puberty, the GnRH neurons become more responsive to neurotransmitter (norepinephrine and dopamine) stimulation. GnRH is released in a pulsatile manner in both male and female animals, which induces a similar pattern of LH and FSH from the pituitary. GnRH levels can be viewed as an indicator of initiation of sexual maturation.

  - **Pituitary (FSH, LH, Prl, TSH).** The gonadotropins FSH, LH, and Prl, secreted by the anterior lobe of the pituitary (hypophysis), are essential in the process of sexual maturation. In the male, LH stimulates testosterone secretion by direct action on the Leydig cells in the testis, and FSH binds to the Sertoli cells within the seminiferous tubules to aid spermatogenesis. FSH also increases the number of LH receptors in the testis, which in turn increases testosterone production and testis growth. Increased prolactin is associated with growth of the prostate and seminal vesicle glands. In the female, FSH and LH act on the ovarian follicular and thecal cells, respectively, to stimulate follicular/oocyte maturation and ovulation. An increase in prolactin levels is essential in determining the acquisition of vaginal opening and the transition to sexual maturity. Thyroid stimulating hormone (TSH) secreted by the pituitary

is the trigger for the release of T3 and T4 (see below), and is essential in the regulation of thyroid activity.

— **Gonads (T, DHT, E2, P).** Androgens are essential in the development of the male reproductive tract, as well as for feedback regulation of the hypothalamic-pituitary axis, sex accessory organ development and maintenance, and spermatogenesis (Goldman et al., 2000; Stoker et al., 2000a). Testosterone (T) and dihydotestosterone (DHT) are the two most active androgens. Testes descent and development; maturation of the epididimydes, vas deferens, seminal vesicles, levator ani/bulbocavernosus; and other aspects of the male reproductive tract, including PPS, are dependent upon T, whereas DHT is responsible for male AGD, the normal regression of nipples, and the development and maintenance of the external genitalia, prostate, and urethra. Estradiol (E2) and progesterone (P) serve similar developmental and maintenance functions in the female.

— **Adrenals (T).**

— **Thyroid (T3, T4).** Thyroid hormones are well known to play essential roles in vertebrate development (Dussault and Ruel, 1981; Myant, 1971; Porterfield and Hendrich, 1993; Porterfield and Stein, 1994; Timiras and Nzekwe, 1989). Experimental work focused on the effects of thyroid hormone on brain development in the neonatal rat supports the concept of a "critical period" during which thyroid hormone must be present to avoid irreversible damage (Timiras and Nzekwe, 1989). Though the duration of this critical period may be different for different thyroid hormone effects, the general view has developed that this is the period of maximal developmental sensitivity to thyroid hormone, and it occurs during the lactational period in the rat (Oppenheimer et al., 1994; Timiras and Nzekwe, 1989). Although thyroid hormone receptors are expressed in fetal rat brains (Bradley et al., 1989; Strait et al., 1990) and thyroid hormone can exert effects on the fetal brain (Escobar et al., 1987, 1988, 1990; Porterfield, 1994; Porterfield and Hendrich, 1992, 1993; Porterfield and Stein, 1994), the lactational period represents a stage of rapid expansion of the thyroid hormone receptors (Perez-Castillo et al., 1985) and an increase in the number of demonstrated effects of thyroid hormone on brain development.

There are a number of endpoints that are sensitive to thyroid hormone agonist/antagonists that may be of use in an *in utero*/lactational exposure study protocol. Growth, body weight, food consumption and efficiency, developmental abnormalities, perinatal mortality, testis size and daily sperm production, vaginal patency, and preputial separation, in addition to thyroid weight and histology, may reflect changes observed in T3 and T4 levels.

3.5 SELECTED STUDIES

The studies discussed below are excellent examples of the use of protocol design to specifically address the issue of endocrine disruption. *In utero*/lactational exposure, reproductive tract morphology, physical landmarks of sexual maturation, sexual behavior, and hormone levels are all skillfully used to systematically identify and characterize the endocrine-disrupting activity of the test compounds. The series of studies on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and di-butyl phthalate elegantly illustrate the sequence of increasingly mechanistic studies, beginning with a basic screening-like study, that can be used to define an endocrine-disrupting compound. Tabular summaries of these and other studies are presented in Tables 4, 5, 6, and 7, which can be found at the end of Section 7.0.

3.5.1. GnRH Modulators

Alteration of GnRH activity, through modulation of receptors or factors associated with release, can affect some indicators of the onset of puberty. For instance, postnatal administration of perphenazine to rats no effect on vaginal patency or age of initial estrus, but caused a delay in the onset of the first regular estrus cycle which persistent after treatment ended (Greenley et al., 1982). Perphenazine also significantly decreased skeletal growth and body weight in both male and female pups (due to decreased food and water intake), decreased serum T3 and T4, and increased prolactin. Female pups also exhibited increased TSH, whereas male pups exhibited decreased FSH and LH. Administration of a GnRH agonist (Fisher et al., 1999) to male rats on pnd 2 or 5 affected development of the reproductive organs, including decreased testis weight after weaning, and alteration of cell development in the efferent duct and rete testis. Testosterone propionate (TP) has been shown to affect the testes and accessory sex organs in male rats (Bari et al., 1984). Administered once to weanling rats, TP only increases ventral prostate weight, presumably because anterior pituitary hormone release has not begun. However, when TP was administered for 15 consecutive days, beginning on pnd 21, testis and accessory sex organ development was adversely affected, including testis, seminal vesicle, and prostate weight, decreased sperm-containing seminiferous tubules, and altered germ cell development. Similar results, with the exception of the alteration in germ cell development, were seen in three- to four-month-old male rats after TP administration.

Atrazine was shown to significantly decrease ventral prostate weight and delay preputial separation at doses that did not affect food consumption (Stoker et al., 2000b). Levels of T, LH, Prl, TSH, T4, and T3 were variable and were not robust indicators of atrazine activity.

3.5.2 Estrogen Modulators

Methoxychlor. Methoxychlor is a DDT derivative that provides an interesting example of the multiplicity of EDC action. Methoxychlor is metabolically activated to several monohydroxy- and dihydroxy metabolites that display estrogenic activity (Bulger et al., 1978). Methoxychlor itself is weakly active or inactive *in vitro* in ER binding and transcriptional activation assays (Maness et al., 1998). HPTE, a methoxychlor metabolite, has high affinity for ERα (Waller et al., 1996a,b). It is an ERα agonist, an ERβ antagonist (Maness et al., 1998), and an AR antagonist. In fact, many natural (i.e., estradiol) and anthropogenic estrogens (o,p DDT) display affinity for AR (Kelce et al., 1995; Waller et al., 1996a,b), acting as AR antagonists and agonists in *in vitro* assays (Danzo, 1997; Sohoni and Sumpter, 1998).

In the female rat, methoxychlor displays estrogenic ERα-mediated activity in many tissues, including the uterus, vagina, brain (behavior), and bone, but not in the hypothalamic-pituitary axis. When administered to the male rat from weaning on, methoxychlor reduces food consumption and growth, delays puberty, reduces sex accessory gland weight, testicular testosterone production *ex vivo*, epididymal size, and epididymal sperm numbers, and stimulates mating behavior (Gray et al., 1989; 1999a). In the male rat, methoxychlor appears to be acting both as an estrogen and as an antiandrogen. However, methoxychlor did not produce any malformations in multigenerational studies (Gray et al., 1989). Unlike estradiol, methoxychlor fails to induce hyperprolactinemia, inhibit LH, or induce pituitary tumors in the male or female rat after long-term high dose treatment (Gray et al., 1988, 1989, 1999a).

Administration of methoxychlor to weanling male rats (50 or 100 mg/kg/day for 20 consecutive days, beginning on pnd 35-36) resulted in decreased final body weight at the lower dose, and decreased body, liver, and combined seminal vesicle weights at the higher dose (Ashby and Lefevre, 2000).

Chapin et al. (1997) evaluated the effect of methoxychlor after administration from gd 17 to pnd 7 (dams) and pnd 7 to pnd 21 or 42 (offspring). Estrogenic activity was observed as accelerated vaginal opening, delayed PPS, disrupted estrous cyclicity, decreased epididymal sperm count, decreased testis, epididymal, seminal vesicle, and prostate weight, and decreased levels of FSH and progesterone.

Diethylstilbestrol (DES). DES, a synthetic nonsteroid estrogen, was evaluated in Donryu rats for adverse effects on the development of female reproductive organs after transplacental exposure (Kitamura et al., 1999). Pregnant rats were given subcutaneous injections of 0.01 or 0.1 mg DES/kg/day on gd 17 and 19. Female offspring were evaluated up to 18 months of age. In the 0.1 mg/kg dose group, decreased mean litter size and shortened pregnancy length were observed. No effect of DES was observed on the body weight and growth of female offspring. However, disrupted estrous cycles (persistent estrus) were observed at a significantly earlier age in the 0.1 mg DES/kg/day treatment group, compared to the controls or the lower dose group. At necropsy, an increased incidence of hypoplasia of the oviduct, cystic dilation of uterus, and small size of the uterine cervix were observed. In addition, increased atrophy of the ovaries, mucification of the vagina, and a dose-dependent increase in endometrial adenocarcinomas were observed in treated offspring. Kledal et al. (2000) conducted a dose range-finding study of DES in mice (four/group), using *in utero* and lactational exposure by subcutaneous injection of the dams with 1, 10, or 100 mg DES/kg/day on gd 6 through weaning. No direct dosing of the pups was used. Although the sample size was too small to allow statistical analysis, examination of the data revealed that the number of uterine implantation sites was noticeably fewer in the high dose group, and no live offspring were produced. There was no effect on gross development of the reproductive tract in either male or female offspring, and males did not exhibit retained nipples. However, histologic examination of the testes of treated males revealed disrupted spermatogenesis in male offspring exposed to 10 µg DES/kg/day, causing disorganized and immature tubules and Leydig cell hyperplasia.

Bisphenol A (BPA). BPA is a high production volume phenol used principally as a monomer in the manufacture of numerous chemical products, including epoxy resins and polycarbonate plastics. Although it had been known for decades that BPA was weakly estrogenic *in vivo* by injection (Dodds and Lawson, 1936), there has been recent, renewed concern over its estrogenicity. The *in vivo* estrogenic potential of BPA was originally demonstrated in short-term uterotrophic assays in rodents using parenteral (injection) administration (e.g., Dodds and Lawson, 1936; Ashby and Tinwell, 1998). Most of the more recent studies utilized oral administration (i.e., dosed feed, dosed water, or oral gavage) but at very high doses (e.g., Morrissey et al., 1987, 1989; Ashby and Tinwell, 1998; Tinwell et al., 2000). Most recently, oral administration of BPA at much lower doses to pregnant rodents has been reported to affect adult male offspring reproductive organ parameters, such as increases in prostate gland weight at 2 and 20 μg/kg/day, increased preputial gland weight at 2 μg/kg/day, and decreased daily sperm production per gram testis (efficiency of daily sperm production) at 20 μg/kg/day in CF-1 mice exposed to BPA in corn oil during prenatal development from gd 11 through 17 by presentation to the dam's buccal cavity (not gavage) (Nagel et al., 1997; vom Saal et al., 1998). The low dose effects of BPA were not replicated in identical study designs with the same and additional lower and higher dose levels and larger numbers of animals per group (Ashby et al., 1999; Cagen et al., 1999a). Colerangle and Roy (1997) administered BPA to Noble rats and reported estrogenic-like stimulation of growth and differentiation of the mammary gland ductal and glandular components. Ashby et al. (2000) repeated this work with BPA and DES, as well as performing the uterotrophic assay in the ovariectomized rat model, and reported no mammary gland stimulation with BPA.

In another recent study, female Wistar rats were exposed to 1 ppm BPA (corresponding to approximately 0.1-0.4 mg/kg/day) in their drinking water for eight to nine weeks (during prebreed, mating, gestation, and lactation), and their adult male offspring were reported to exhibit significantly reduced testes weights (Sharpe et al., 1995). This study could not be replicated by the original authors (Sharpe et al., 1998) who still stand by their original findings, nor by another larger study employing a larger number of dose groups, more animals per dose, and more reproductive parameters, but the same study exposure route, timing, duration, and strain of rat (Cagen et al., 1999b). The question of the reality and reproducibility of the low-dose effects of BPA still remains.

The question of qualitatively and quantitatively different responses to different routes of BPA administration, with oral administration requiring much higher doses to effect than parenteral subcutaneous administration (e.g., Twomey, 1998a,b; Kwon et al., 2000), was resolved by work by Pottenger et al. (2000). Pottenger et al. (2000) showed that there was a clear route dependency in the toxicokinetics and metabolism of ^{14}C-labeled BPA after a single oral, intraperitoneal (ip), or subcutaneous (sc) dose of either 10 or 100 mg/kg to Fischer 344 rats. The relative bioavailability of BPA and the plasma radioactivity was markedly lower after oral administration as compared to sc or ip administration, thus providing an explanation for the apparent route differences in estrogenic potency observed for BPA in rats (Pottenger et al., 2000).

The study recently completed (Tyl et al., 2001a,b) evaluated exposure of CD® (Sprague-Dawley) rats to BPA administered in the diet *ad libitum* at 0, 0.015, 0.300, 4.50, 75.0, 750, and

7500 ppm for three generations, one litter per generation. These dietary levels provided approximate intakes of 0, 1 µg/day (0.001 mg/kg/day), 20 µg/kg/day (0.020 mg/kg/day), 250 µg/kg/day (0.250 mg/kg/day), and 5, 50, and 500 mg/kg/day, respectively. The wide dose range for this study was selected to evaluate the potential reproductive toxicity of BPA under standard EPA test guidelines, as well as attempt to replicate published low dose effects of BPA in rats and mice (see above). The interpretation of the results of this study, therefore, required not only the typical analysis of biological relevance of any effects in treated animals at the high dose in relationship to any effects observed at lower doses, but also careful consideration of potential estrogen-like effects resulting from receptor-based events which have been hypothesized by some investigators (vom Saal et al., 1998) as possible at lower doses. As a result of these purported effects, additional assessments were added (beyond the guideline requirements) for this study, including a third generation, six dose groups from 0.015 to 7500 ppm (plus control, 0 ppm), examination for retained nipples and areolae in F1, F2, and F3 preweanlings, retention of F3 offspring (30/sex/group) until adulthood with exposure continuing, with anogenital distance and acquisition of puberty in both sexes, with epididymal sperm and testicular spermatid measurements in the retained F3 males, estrous cyclicity in retained F3 females, and full necropsy and histopathology of the retained F3 adults.

As noted earlier, BPA has been shown to have weak estrogenic activity in some screening assays. The Tyl study (Tyl et al., 2001a,b) evaluated a number of reproductive endocrine sensitive endpoints, including preputial separation, vaginal patency, anogenital distance, estrous cyclicity, ovarian follicle counts, sperm and spermatid assessments, and reproductive organ weights and histopathology. The results from the dietary 17-β-estradiol (E2) study by Biegel et al. (1998a,b) provide a standard for evaluating "estrogen-like" responses. Based on the kinetic information for BPA and 17-β-estradiol, the bioavailable dose should be similar at comparable dietary concentrations, and BPA is generally reported to be approximately 15,000 fold less potent than 17-β-estradiol from *in vitro* receptor binding/transcriptional activation screening assays (Gaido et al., 1997) and 10,000 fold less potent from *in vivo* subcutaneous injection in mice (Milligan et al., 1998). Therefore, if BPA were estrogenic, it would be expected that doses of 750 ppm or greater should result in effects similar to those described above for 17-β-estradiol at 0.05 ppm or greater. However, only two of the estrogen-specific changes, reduced live litter size at birth and reduced paired ovary weights, were observed in this study and only at 7500 ppm. This dietary dose also produced evidence of profound parental toxicity and may have exceeded the maximum tolerated dose (MTD) based on body weight and weight gain effects. The identification of these estrogen-specific endpoints affected only at 7500 ppm supports the designation of BPA as a very weak estrogen only at very high doses ($\geq$ MTD) by oral administration. No estrogenic effects were observed at any doses below 7500 ppm in this study.

In the Tyl study (Tyl et al., 2001a,b), age at acquisition of preputial separation was significantly delayed for F1, F2, and F3 males at 7500 ppm using ANCOVA with body weight at acquisition as the covariate. Age at acquisition of vaginal patency was also significantly delayed for F1, F2, and F3 females at 7500 ppm using ANCOVA with body weight at acquisition as the covariate. Body weight at acquisition was significantly reduced for F1 males and females and for F2 and F3 males only. In this study, the lower body weight of the F1, F2, and F3 offspring at 7500 ppm presumably resulted in the observed delay in acquisition of vaginal patency and

preputial separation. Since acquisition of both landmarks in both sexes of both generations was delayed, these results are not considered to be due to estrogen receptor-mediated events or other endocrine-related toxicity.

The significant effect on acquisition of reproductive landmarks in F1 and F2 offspring required measurement of anogenital distance in newborn F2 and F3 offspring, as specified in the guidelines (U.S. EPA, 1998b). Anogenital distance on pnd 0 in the newborn F2 males was equivalent (statistically and biologically) across all groups, with F3 male pup body weights per litter also statistically equivalent across all groups. Anogenital distance in the newborn F2 females was significantly longer at 0.015, 0.300, 4.50 (not 75.0), and 750 (not 7500) ppm, with mean values of 0.98, 0.98, 0.98, and 0.99 mm at 0.015, 0.300, 4.50, and 750 ppm, respectively, relative to the control group mean value of 0.98 mm. These effects represent an increase in anogenital distance of 0.03, 0.03, 0.03, and 0.04 mm, equivalent to 3.16, 3.16, 3.16, and 4.21% increases at 0.015, 0.300, 4.50, and 750 ppm, respectively. F3 female pup body weights per litter were statistically equivalent across all groups. Anogenital distance is under androgenic control (Gray et al., 1998; Gray and Ostby, 1998) and does not appear to be affected by estrogens (Biegel et al., 1998a), but is affected by pup body weights (Ashby et al., 1997a). For example, recent data (Gallavan et al., 1998) from 1501 control CD® rats indicated that a one gram increase in body weight leads to a 0.19 mm increase in anogenital distance. In this study, all female anogenital distance values round to 1.00 mm (with historical control values in the performing laboratory from two recent previous studies of 0.73 ± 0.03 [SE] and 0.76 ± 0.02 mm in F2 female pups on pnd 0, with mean body weights of 6.08 ± 0.11 and 5.93 ± 0.11 g). These data suggest that very small increases in anogenital distance in newborn females, as seen in this study (and in the octylphenol study; see above), are of no biological significance.

The conclusions of this study of dietary exposure of CD rats to BPA *ad libitum* for three generations, one litter per generation, at 0, 0.015, 0.300, 4.50, 75, 750, and 7500 ppm are shown in Table 3.

Table 3. Results of the Multigeneration Study of BPA (Tyl et al., 2001a,b)

- Decreased adult body weights and weight gains at 750 and 7500 ppm for F0, F1, and F2 parents and F3 retained adults
- Offspring toxicity (reduced body weights during lactation) beginning on pnd 7 at 7500 ppm for F1, F2, and F3 offspring
- No consistent or dose-related effects on anogenital distance on pnd 0 for F2 and F3 offspring
- Delayed acquisition of vaginal patency and preputial separation for F1, F2, and F3 offspring at 7500 ppm; considered related to reduced body weight
- No effects on reproductive parameters for any generation
- Significant reductions in total and live pups per litter at birth on pnd 0 (and on pnd 4 precull) at 7500 ppm for F1, F2, and F3 generations in the absence of any increased prenatal (postimplantation) loss, in the presence of substantial maternal systemic toxicity

- Significant reductions in absolute and relative paired ovary weights at 7500 ppm in F1, F2, and F3 (absolute only) females, in the presence of substantial maternal systemic toxicity
- No effects on testes weights or histopathology in any generation
- No effects on epididymal sperm counts, motility, or morphology in any generation
- No consistent effects on testicular homogenization-resistant spermatid head counts, daily sperm production (reduced only in F2 males), or in efficiency of daily sperm production
- Only two estrogen-like effects were observed--reduced ovarian weights and reduced litter size at birth--only at 7500 ppm, which was at or above the MTD
- No effects on reproductive organ weights or histopathology for F0, F1, F2, or F3 male or female adults
- Histopathologic findings only in females in F0, F1, and F2 (but not F3) generations, with increased incidences of renal tubular degeneration in the kidneys and chronic hepatic inflammation in the livers only at 7500 ppm
- No treatment- or dose-related increases in retained nipples and/or areolae in preweanling males at any dose for F1, F2, and F3 generations
- No consistent differences between high dose and control (ten females/group) paired ovarian primordial follicle counts; significantly increased at 7500 ppm for F0 females, unaffected for F1, F2, and F3 females

The only two "estrogen-like" responses observed, reduced absolute and relative paired ovarian weights and reduced total and live pups per litter at birth with no increase in prenatal (postimplantation) loss, were present only at 7500 ppm, a dietary dose which was at or above the parental MTD in this study.

The NOAEL for adult systemic toxicity was 75 ppm (approximately equivalent to 5 mg/kg/day); the NOAELs for reproductive and postnatal toxicity were 750 ppm (approximately equivalent to 50 mg/kg/day) in CD® (Sprague-Dawley) rats under the conditions of this study.

In agreement with the results of Tyl et al. (2000), Kwon et al. (2000) administered BPA by gavage to pregnant CD® (SD) rats at 0, 3.2, 3.2, or 320 mg/kg/day from gd 11 through pnd 20. DES at 15 µg/kg was employed as a positive control. (The DES dose was based on the relative *in vitro* estrogenic potency of DES versus BPA, in which a DES dose of 15 µg/kg was equivalent to BPA at 320 mg/kg in *in vitro* binding affinity for both ERα and Erβ). Offspring female pubertal development was unaffected by indirect BPA exposure at any dose. There were also no effects on the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) of the brain in ten-day-old offspring females, or on estrous cyclicity or sexual behavior of the offspring females at four months of age, and no effects on offspring male reproductive organ weights at six months of age, including testis, epididymis, seminal vesicle, ventral and dorsolateral prostate lobes. DES increased the volume of the SDN-POA in offspring females and caused irregular estrous cyclicity (Kwon et al., 2000).

The mechanism of action of BPA (at high doses and/or administered parenterally) is not known. BPA is not structurally similar to E2, but its two hydroxy phenolic ring structures resemble the synthetic estrogen DES. BPA also contains a hydroxy group in the C3 position which is required for ER binding (Chun and Gorski, 2000; p. 161).

Additionally, the interaction of BPA with the estrogen receptor alpha (ERα) was examined in the human HepG2 hepatoma cell line with luciferase (reporter gene) and in the immature rat uterotrophic assay (Gould et al., 1998). BPA was 26-fold less potent than E2 (approximately 70% of the E2 activation) in activating ER-WT (wild type) and was a partial agonist (inducing approximately 40% of the E2 activity) with ERα. The use of ERα mutants in which the AF1 or AF2 regions were inactivated allowed classification of ER ligands into mechanistically distinct groups. The pattern of BPA activity with the ERα mutants differed from the activity observed with E2 with estrone and estriol (weak estrogens), with raloxifene or 4-OH-tamoxifen (partial ERα agonists), or with ICI 182,780 (a pure antagonist). In the immature rat uterotrophic assay, female Sprague-Dawley rats were dosed by oral gavage with BPA alone (at 0, 5.0, 10, 25, 50, 100, or 150 mg/kg/day) or with BPA and 0.5 µg/day of E2 in corn oil by intraperitoneal injection for three days (pnd 21-23) and killed 20 hours after the last treatment. BPA alone did not affect uterine weight, but it did increase uterine peroxidase and progesterone receptor (PR) levels. E2 alone did increase uterine weight and did increase uterine peroxidase and PR levels to a greater extent than did BPA. Following simultaneous administration of BPA and E2, BPA antagonized the stimulatory effects of E2 on peroxidase activity and PR levels but did not inhibit E2-induced increased uterine weight. The authors concluded that "These results demonstrate that BPA is not merely a weak estrogen mimic but exhibits a distinct mechanism of action at the ERα" (Gould et al., 1998; p. 203). They continue "The distinct activity of BPA is most likely due to an induction of a conformation of the activated ERα by BP that differs from these other known classes of ER ligands. Thus, it is likely that BPA will induce a unique subset of ERA-responsive genes *in vivo* resulting in a biological response which differs from the known classes of ER ligands" (Gould et al., 1998; pp. 209-210).

In fact, there is still controversy whether BPA induces a uterotrophic response in ovariectomized mice and whether a known "pure" anti-estrogen (ICI 182,780) completely blocks this response (Papaconstantinou et al., 2000). The BPA doses used were 0.8, 2, and 8 mg/day (in a 25 g mouse, equivalent to approximately 32-320 mg/kg/day). The implication for this very recent discussion is whether the low submaximal uterotrophic response of BPA is ER independent (Safe, 2000). Safe (2000, p. 252) concludes that "this is another example of divergent effects observed for same estrogenic compound in comparable assays, and the explanations for these differences are currently lacking. These present, apparent discrepancies make the assessments of xenoestrogenic compounds extremely controversial while concomitantly highlighting the need to resolve these potentially important public health concerns."

In summary for the phenols, in all of the three multigeneration studies, the findings from short-term *in vitro* and *in vivo* screens at high doses and inappropriate routes of administration were <u>not</u> confirmed: the effects initially reported for octylphenol (OP) and BPA under those artificial conditions were not substantiated. Only the acceleration of acquisition of vaginal patency in F1, F2, and F2 offspring females (and the increased uterine weights on pnd 21 in F1

females only) at 650 and 2000 nonylphenol (NP), and the reduced ovarian weights and reduced litter size in F1, F2, and F3 generations at 7500 ppm BPA, implied some estrogen-like effects. The effects from (NP) on F2 sperm and spermatid number (in the absence of any effects on sperm motility or morphology, of effects on reproductive organ weights or histopathology, or of effects on reproductive or postnatal offspring development) are unexplained; the authors "feel it is unwarranted to conclude that this study shows an adverse effect of a mild estromimetric on male reproductive development" (Chapin et al., 1999, p. 89). The "estrogen-like" effects from BPA at 7500 ppm (approximately 500 mg/kg/day) occurred only in the presence of many other indicators of parental systemic toxicity (≥MTD) and may also not indicate an adverse effect of a mild "estromimetic."

Aroclor. The estrogenic effects of the aroclor family of polychlorinated biphenyls (PCBs) administered to female rats either on pnd 2-3 or on pnd 21, was evaluated using uterine weight, day of vaginal opening, estrous cyclicity, ovarian, uterine, adrenal, and anterior pituitary weight, and microscopic examination of the ovaries (Gellert, 1978). Evaluation of these parameters served to distinguish between the estrogenic potencies of several PCBs. After perinatal administration, Arochlor 1221 accelerated vaginal opening, persistent estrus, and an increase in anovulatory animals, mimicking premature reproductive aging were observed, whereas the other PCBs tested had no effect on these parameters. Administration at weaning had little or no effect.

Tween 80. Estrogenic effects were observed in female rats after administration of 10% Tween 80 (Gajdova et al., 1993). Neonatal female rats injected with 1, 5, or 10% aqueous Tween 80 on pnd 4-7 exhibited accelerated maturation. Vaginal opening of all treated rats was significantly sooner than the controls (pnd 15-16 vs. pnd 25-27). In addition, the estrous cycle was prolonged and the animals exhibited persistent vaginal estrus. Animals were below expected weight, as were the uterus and ovaries. However, the adrenal glands and hypophysis weights were increased compared to the control animals. Histological examination of the ovaries, of animals treated with 10% Tween 80, revealed degenerative follicles and a lack of corpora lutea; the uteri of these treated animals exhibited metaplasia of the endometrium. Changes observed were indicative of the inhibitory activity of estrogen on growth (through depressed function of the anterior pituitary and inhibition of growth hormone) and chronic exposure to estrogens.

Raloxifen hydrochloride. The Segment II/III study of orally administered raloxifen hydrochloride, conducted by Buelke-Sam et al. (1998), illustrates the use of selected parameters in the evaluation of estrogenic effects of a nonsteroidal compound after *in utero*/lactational exposure. Raloxifen is a selective nonsteroidal estrogen receptor modulator and binds to the estrogen receptor with an affinity somewhat less that estrogen. Thus, it would be expected to disrupt estrogen-dependent processes during development and maturation. Raloxifen exposure resulted in decreased maternal weight and food consumption during gestation, lengthening of the gestational period, altered parturition (resulting in increased maternal and pup mortality), depressed offspring body weights during lactation, accelerated development of negative geotaxis and incisor eruption, delayed eye opening, and accelerated vaginal opening, all consistent with estrogen antagonist activity.

3.5.3 Androgen Modulators

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin. Recent literature contains a number of well-conducted studies describing the endocrine-disrupting effect of the polyhalogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Mably et al., 1991, 1992a, b, c; Bjerke and Peterson, 1994; Bjerke et al., 1994a, b; Roman et al., 1995; Chaffin et al., 1996; Chaffin and Hutz, 1997;; Sommer et al., 1996; Flaws et al., 1997; Gray and Ostby, 1995; Gray et al., 1995; 1997a, b; Wolf et al., 1999; Theobald and Peterson, 1997; Cooke et al., 1998; Roman and Peterson, 1998; Roman et al., 1998; Loeffler and Peterson, 1999; Hamm et al., 2000). The bulk of the work originates from the laboratory of Dr. Richard E. Peterson, University of Wisconsin, Madison, WI (Mably et al., 1991, 1992a, b, c; Bjerke and Peterson, 1994; Bjerke et al, 1994a, b; Roman et al., 1995; Chaffin et al., 1996; Sommer et al., 1996; Flaws et al., 1997; Theobald and Peterson, 1997; Roman and Peterson, 1998; Roman et al., 1998; Loeffler and Peterson, 1999; Peterson et al., 1992), and all describe results in rats, with the exception of Theobald and Peterson (1997), which describes work in mice. All the rat studies employed a single oral dose of TCDD (1.0 microg/kg or less) or vehicle (corn oil/acetone 19/1 v/v, 2 ml/kg) on gd 15. Mice were dosed on gd 14 with 15-60 microg/kg TCDD or vehicle (corn oil/acetone, 95/5 v/v, 5 ml/kg). Gd 15 or 14 was selected because most of the organogenesis was complete, and the hypothalamic-pituitary axis was in place. Because of the ability of TCDD to be sequestered in the maternal body fat, a single maternal dose late in gestation provides lactational exposure as well through the milk (Van Den Berg et al., 1987). The studies looked primarily at the effects and mechanisms of TCDD toxicity in the male, focusing on the prostate, sexual behavior, spermatogenesis and male reproductive capacity, and hormone responsiveness, although several studies (Gray and Ostby, 1995; Gray et al, 1997b; Flaws et al., 1997; Wolf et al., 1999) looked at female genital dymorphogenesis.

Roman et al. (1998) evaluated the effect of 1.0 µg TCDD/kg on the development of the prostate. On gd 20, male fetal rats were examined for prostate development. TCDD interfered with the initial outgrowth of prostatic tissue. Examination of males up to pnd 21 showed decreased differentiation and cell proliferation, histological rearrangement, and a decrease in androgen receptor-positive cells in the prostatic tissue. Other studies using doses of TCDD of 0.7 or 1.0 µg/kg showed that the prostate was uniquely susceptible to the adverse effects of TCDD, especially at early postweaning ages (Bjerke et al., 1994a; Roman et al., 1995), that the effect after *in utero*/lactational exposure was not due to a decrease in testicular androgen production or prostate DHT concentration (Roman et al., 1995), or a direct effect on prostatic gene transcription (Roman and Peterson, 1998), and that testosterone response of the prostate in adulthood was decreased as a result of *in utero*/lactational exposure (Bjerke et al., 1994a). In another set of studies, Mably et al. (1991, 1992a, b, c) looked at the effect of *in utero*/lactational exposure to TCDD (0.064-1.0 µg/kg) on male reproductive tract development, androgenic status, reproductive function, sexual behavior, testicular histology, and fertility. Their results indicate that TCDD, at doses as low as 0.16 µg/kg on gd 15, caused a significant dose-related decrease in AGD on pnd 1 and 4, and delays in testicular descent. Testis, epididymis, cauda epididymis, seminal vesicle, and prostate weight were significantly decreased on pnd 32, 49, 63, and 120. In addition, sperm production on pnd 49, 63, and 120 were decreased, although morphology and motility were unaffected. Testosterone and DHT levels were not significantly affected. Sexual behavior and LH response of prenatally exposed males showed evidence of feminization, which

the authors concluded was most likely the result of impaired sexual differentiation in the CNS. However, Bjerke et al., (1994b) showed that feminization of male sexual behavior was not due to alterations in estrogen receptor binding or volume of sexually differentiated nuclei in the brain. Sommer et al. (1996) showed that TCDD exposure on gd 15 (1.0 µg/kg) caused decreased daily sperm production, and may cause increased sperm phagocytosis in the excurrent duct system. However, these effects were not observed until pnd 92-93. Not all of these effects have been reproducible.

Flaws et al. (1997), Gray and Ostby (1995), and Gray et al. (1997b) showed that 1.0 µg TCDD/kg induced cleft clitoris and thickened vaginal threads partially occluding the vaginal opening in female rats. Female hamsters, treated by gavage on day 11 of gestation with 2 µg TCDD/kg, also display clitoral clefting and reduced fertility as a result of several functional reproductive problems, but they did not display the vaginal thread (Wolf et al., 1999). In male rat and hamster offspring, treated by gavage on day 11 of gestation with 2 µg TCDD/kg, puberty was delayed, ejaculated and epididymal sperm numbers were reduced, while the reductions in ventral prostate, seminal vesicle, and testis size, displayed during peripubertal life (49-63 days of age), were attenuated with age (Gray et al., 1995). Mating behavior was normal in male hamsters, while male rats had some difficulty achieving intromissions (insertion of penis into vagina; copulation). No malformations were noted in male rat or hamster offspring at these dosage levels.

In an effort to separate the effects of *in utero* exposure from those of lactational exposure, Bjerke and Petersen (1994) treated pregnant rats with 1.0 µg/kg TCDD on gd 15, then cross-fostered the pups on pnd 1 to vehicle or TCDD-treated mothers. Thus, four exposure groups were formed: nonexposed pups, pups exposed *in utero* only, pups exposed during lactation only, and pups exposed both *in utero* and during lactation. The pups were evaluated for AGD, testis descent, PPS, plasma testosterone, testis, glans penis, epididymides, prostate and seminal vesicle weight, daily sperm production, cauda epididymal sperm reserves, and sexual behavior during postnatal and early adult development. The authors concluded that all of the endpoints could be characteristically affected by exposure to TCDD *in utero* only, with the exception of feminization of male sexual behavior, which required lactational exposure. In addition, effects on daily sperm concentration and PPS required *in utero* exposure. These results were significant since only 0.1% of the maternal dose reaches the fetus transplacentally, whereas more than 7% of the dose has been shown to accumulate in each pup following lactational exposure (Nau and Bass, 1981; Van Den Berg et al., 1987). Studies conducted in mice by this laboratory showed that the mouse is not as sensitive as the rat to the effects of TCDD (Theobald and Peterson, 1997). In mice exposed to 15, 30, or 60 µg TCDD/kg on gd 14, eye opening, ventral prostate, coagulating gland, thymus, and pituitary weight were the most sensitive endpoints in male offspring, exhibited as decreases at the lowest dose. Female offspring exhibited only a slight decrease in uterine weight at the highest maternal dose.

Other studies of TCDD effects after maternal rat exposure on gd 15 were associated with impaired postnatal development of the seminal epithelium (Hamm et al., 2000) and were not associated with decreased steroidogenic enzyme activity in the male reproductive tract (Cooke et al., 1998). In female offspring, decreased circulating levels of estrogen may be the result of alterations of estrogen receptor mRNA or estrogen receptor DNA-binding activity in estrogen-

sensitive tissues (Chaffin et al., 1996b). Loeffler and Peterson (1999) showed that TCDD and another androgen modulator, DDE, exerted additive effects, probably by different mechanisms. Treatment with one or both agents during gestation affected prostate weight and caudal epididymal sperm number, but did not affect anogenital distance, age at onset of puberty, daily sperm production, and testicular and nonprostate accessory organ weights. DDE caused retained nipples in offspring males.

Vinclozolin. The cellular and molecular mechanisms of action of the fungicide vinclozolin, an antiandrogenic EDC, are among the most thoroughly characterized for this class of compound. Vinclozolin metabolites, M1 and M2, but not vinclozolin itself, competitively inhibit the binding of androgens to the mammalian androgen receptors (AR). M1 and M2 also inhibit DHT-induced transcriptional activity in cells transfected with the human AR. Kelce et al. (1997) demonstrated that vinclozolin treatment altered gene expression *in vivo* in an antiandrogenic manner. In contrast to their ability to bind to the androgen receptor, neither vinclozolin nor its antiandrogenic metabolites display affinity for the estrogen receptor, although they do have weak affinity for the progesterone receptor. Vinclozolin, M1, and M2 do not inhibit 5α-reductase activity *in vitro*, the enzyme required for the conversion of T to the more active androgen DHT. Androgen-induced gene expression is a multistep process. Agonist-bound AR undergoes conformational changes, loses heat-shock proteins, is imported from a perinuclear region into the nucleus, forms homodimers, and binds androgen response elements on regulatory sequences on the DNA "upstream" from androgen-responsive genes, activating mRNA synthesis. Binding of antagonists to AR results in conformations that differ from that obtained with the natural ligands, such that AR-DNA binding and gene expression are blocked. In addition, vinclozolin inhibits growth of androgen-dependent tissues in the castrated, immature, testosterone-treated, and pubertal male rat. In the intact pubertal and adult male rat, vinclozolin treatment also alters hypothalamic-pituitary-gonadal function. Oral treatment with vinclozolin causes elevations of serum LH and testosterone.

Two studies of the effect of the fungicide vinclozolin on male rats used both physical landmarks and receptor function to define the anti-androgenic effect of this compound (Gray et al., 1994; Monosson et al., 1999). Gray et al. (1994) administered vinclozolin in corn oil orally (0, 100, or 200 mg/kg/day) to pregnant rats during the period of fetal/pup sexual differentiation (i.e., gd 14-pnd 3). Male offspring were examined for evidence of demasculinization. At birth, AGD was reduced, and nipple retention was prominent on pnd 14. All treated male offspring had cleft phallus with hypospadias. After puberty, sexual behavior was feminized, with most offspring unable to achieve intromission. Those offspring that appeared to achieve intromission were unable to ejaculate properly, probably due in part to penile malformations. At necropsy at one year, numerous malformations of the reproductive tract were observed, including ectopic scrota/testes, vaginal pouch, and small to absent sex accessory glands, particularly the prostate. These males also had renal lesions. Since the female offspring did not exhibit signs of estrogenic-like alterations, and since the effects seen in the male offspring were nearly identical to those seen with flutamide, the authors concluded that vinclozolin exerted its male effects as an androgen-receptor antagonist. In a subsequent study, vinclozolin was orally administered (0, 10, 30, or 100 mg/kg/day) to male rats from pnd 22 until termination on pnd 54 or 56 (Monosson et al., 1999). Vinclozolin at 30 and 100 mg/kg/day delayed PPS and retarded sex accessory gland and epididymal growth. At necropsy, serum LH, testosterone, and DHT levels were increased at

the high dose. Testis size and sperm production were unaffected. Analysis of the subcellular distribution of the androgen receptor suggested that vinclozolin metabolites acted by tying up the androgen receptor, preventing it from interacting with DNA, and thus altering gene expression and protein synthesis. Ashby and Lefevre (2000) observed decreased combined epididymide weight after a 14-day administration of 100 mg/kg/day vinclozolin to weanling rats, beginning on pnd 22-23 or 35-36.

A series of studies have described the dose-dependent effects of vinclozolin (Kelce et al., 1994; Gray et al., 1999b; Wolf et al., 2000). Oral administration of vinclozolin at 100 or 200 mg/kg/d to pregnant rats during sexual differentiation (gd 14 to pnd 3) demasculinizes and feminizes the male offspring. Vinclozolin-treated male offspring display female-like anogenital distance (AGD) at birth, retained nipples, cleft phallus with hypospadias, suprainguinal ectopic testes, a blind vaginal pouch, epididymal granulomas, and small to absent sex accessory glands. In contrast, the testis is a relatively insensitive target for antiandrogens as compared to the external genitalia and sex accessory glands, and female offspring do not display malformations or permanent functional alterations. A comparison of the *in vitro* dosimetry data with the biological effects of vinclozolin revealed that when M1 and M2 concentrations in maternal serum approach their respective K_i values for AR, male offspring displayed hypospadias (Kelce et al., 1994).

When vinclozolin was administered by gavage to the dam at 0, 3.125, 6.25, 12.5, 25, 50, or 100 mg/kg/d, from gd 14 to pnd 3 (Gray et al., 1999a), doses of 3.125 mg/kg/d and above reduced anogenital distance in newborn male offspring and increased the incidence of nipples/areolas in infant male rats. These effects were associated with permanent alterations in other androgen-dependent tissues. Ventral prostate weight in one-year-old male offspring was reduced in all treatment groups (significant at 6.25, 25, 50, and 100 mg/kg/d), and permanent nipples were detected in males at 3.125 (1.4%), 6.25 (3.6%), 12.5 (3.9%), 25 (8.5%), 50 (91%), and 100 (100%) mg/kg/d. The authors indicated that permanent nipples in adult male offspring (not to be confused with areolas or what some authors incorrectly described as "retained nipples" in infant male rats) have never been observed in a control male from any study in their laboratory. Vinclozolin treatment at 50 and 100 mg/kg/d induced reproductive tract malformations and reduced ejaculated sperm numbers and fertility. Even though all of the effects of vinclozolin likely resulted from the same initial event (AR binding), the different endpoints displayed a wide variety of dose-response curves and ED50s, and some of these dose-response curves failed to display an obvious threshold.

In a study designed to determine the most sensitive period of fetal development to the antiandrogenic effects of vinclozolin, pregnant rats were dosed orally with 400 mg vinclozolin/kg/d in corn oil on either gd 12-13, 14-15, 16-17, 18-19, or 20-21 (Wolf et al., 2000). Malformations and other effects were seen in male rat offspring dosed with vinclozolin on gd 14-15, 16-17, and 18-19, with the most pronounced effects resulting from exposure on gd 16-17. These effects include reduced AGD, increased number areolas and nipples, malformations of the phallus, and reduced levator ani/bulbocavernosus weight. The fetal male rat is most sensitive to antiandrogenic effects of vinclozolin on gd 16 and 17, although effects are more severe after administration of vinclozolin from gd 14 through 19, with 100% of male offspring affected.

These data demonstrate that vinclozolin produces subtle alterations in sex differentiation of the external genitalia, ventral prostate, and nipple tissue in male rat offspring at dosage levels below the previously described no-observed-effect-level (NOEL). Some of the functional and morphological alterations were evident at dosage levels below that required to induce malformations and reduce fertility. Hence, multigenerational reproduction studies of antiandrogenic chemicals conducted under the "old" multigenerational test guidelines that did not include endpoints sensitive to antiandrogens at low dosage level could yield a NOEL that is at least an order of magnitude too high.

p,p' DDE and DDT. In 1995, Kelce et al. reported that p,p-DDE displayed antiandrogenic activity both *in vivo* and *in vitro* that was similar to vinclozolin, both being AR antagonists. *In vitro*, p,p-DDE binds to the AR and prevents DHT-induced transcriptional activation in cells transfected with the human AR and inhibits androgen-dependent gene expression *in vivo* (Kelce et al., 1997). Interestingly, Wakeling and Visek (1973) reported that several chlorinated pesticides including dieldrin and o,p' DDT inhibited binding of DHT to proteins in the rat prostate cytosol (they did not examine p,p' DDT or DDE).
When p,p' DDE is administered by gavage in oil during gestation treatment at 100 mg/kg/d (days 14-18 of gestation) it reduces AGD and induces hypospadias, retained nipples and smaller androgen-dependent tissues in treated Long Evans Hooded (LE) and Sprague-Dawley (SD) male rat offspring (Gray et al., 1999c). While the alterations were evident in both rat strains, the SD strain appeared to be more affected. Only the SD strain displayed hypospadias and other effects were of a greater magnitude in the treated SD than in the LE rats. It is uncertain if this reflects a true strain difference in sensitivity or if it merely results from experiment to experiment variation. You et al. (1998) studied the effects of p,p'-DDE on the male offspring using the same protocol and they also found that p,p'-DDE induced antiandrogenic effects on AGD and areola development in both LE and SD rat strains. Following oral treatment with p,p' DDE at 100 mg/kg/d as above, fetal rat tissue p,p' DDE levels ranged from 1 to 2 μg/g during sexual differentiation in this dosage group (You et al., 1999) a concentration well below that seen in human fetal tissues from the late 1960s in areas of normal DDT use in the USA and Israel (Wassermann et al, 1967; Curley et al., 1969).

In the rabbit (Dutch Belted), when p,p-DDT, which also is antiandrogenic (Kelce et al., 1995), was administered during gestation (does treated) and lactation (pups treated) at dosage levels of 25 mg/kg: dams; 10 mg/kg: pups; 1 time/week, reproductive abnormalities were displayed by male offspring (Veeramachanei et al., 1995). Infantile exposure alone resulted in delays in testicular descent in the rabbit, while combined lactational plus gestational exposure induced uni/bilateral cryptorchidism. Serum levels of p,p' DDT and DDE in offspring were 208 ppb p,p-DDT and 38 ppb p,p-DDE. These levels are well below the concentrations of p,p-DDT and DDE seen in human fetal tissues in the USA, in areas of normal pesticide use during the late 1960s (Curley et al., 1969). Taken together, these data indicate that adverse developmental reproductive effects are seen in rats and rabbits at levels (based on tissue residues) that are within the range reported for the human fetus in the late 1960s, exposed to DDT at this time through legal applications (Wassermann et al., 1967; Curley et al., 1969).

Phthalate Esters. Recently, concerns about exposures of children to phthalates in toys and other products have resulted in a ban in phthalates in certain toys by the European Union.

Although industry has repeatedly assured the safety of these chemicals (Koop and Juberg, 1999), most of them, including diethylhexyl phthalate (DEHP) have never been rigorously examined by the manufacturers for multigenerational effects.

The phthalates represent a class of toxicants which alter male reproductive development via a mechanism of action that does not appear to involve AR or ER binding. Although many of the same effects are seen in animals exposed *in utero* to AR antagonists, like vinclozolin, *in vitro* studies, conducted to determine the biochemical mechanism responsible for the adverse developmental effects of DEHP, found that neither DEHP nor the primary metabolite MEHP compete with androgens for binding to the androgen receptor (Parks et al., 2000).

The male reproductive tract also appears to be the primary target for the endocrine-disrupting activity of the plasticizer di-n-butyl phthalate (DBP). Studies by Ema et al. (2000), Mylchreest et al. (1998, 1999, 2000), and Gray et al. (1999c) evaluated the effects of DBP administered orally to pregnant rats during gestation, or during gestation and lactation, on the postnatal sexual development of male offspring. Mylchreest et al. (1998) administered DBP (250, 500, or 750 mg/kg/day) or vehicle (corn oil) from gd 3 through pnd 20, with a two-day interruption for parturition (pnd 1-2). Evaluation of both male and female offspring was conducted up until pnd 20. DBP had little effect on female reproductive development. Anogenital distance, vaginal opening, first estrus, length of estrous cycle, or frequency of cornified smears were not significantly affected. Body, liver, kidney, adrenal, ovary, and uterus weights (in those females with morphologically intact uteri) were unaffected. Male pups had decreased anogenital distance at 500 and 750 mg/kg/day, but not at 250 mg/kg/day. At sexual maturity, males of treated mothers exhibited a dose-dependent increase in malformations of the genital tract, including hypospadias, reduced penis size, under-developed or absent epididymis with atrophy of the ipsilateral testis, and degeneration of the seminiferous tubules. Nonscrotal testes were observed at all dose levels, with most nonscrotal testes ectopic and atrophied upon examination. PPS was unaffected in pups with normal penis development. At 500 and 750 mg/kg/day DBP, the prostate and seminal vesicles had reduced weight or were underdeveloped. Body, liver, and adrenal weights were comparable in treated and control pups. Some renal lesions were observed.

In a subsequent study (Mylchreest et al., 2000), pregnant rats were given 0.5-500 mg/kg/day on gd 12-20. Male offspring were evaluated to pnd 110. Results were similar to those reported in Mylchreest et al. (1998). Retained nipples were observed at $\geq$100 mg/kg/day, whereas decreased AGD and malformations were observed at 500 mg/kg/day. This study established a No-Adverse-Effect-Level (NOAEL) at 50 mg/kg/day, and a Low-Adverse-Effect-Level (LOAEL) at 100 mg/kg/day, based on nipple retention.

While some have suggested that di-n-butyl phthalate (DBP) was estrogenic (Jobling et al., 1995), this activity is only displayed *in vitro*. For example, di-n-butyl phthalate (DBP) did not produce any signs of estrogenicity in the ovariectomized female rat (Gray et al., 1999c). DBP (sc at 200 or 400 mg/kg/d or by gavage at 1000 mg/kg/d, administered for two days, followed on the third day by 0.5 mg progesterone sc) did not induce a uterotropic response or estrogen-dependent sex behavior (lordosis). In addition, phthalate-treatment did not increase uterine weight (Meek et al., 1997) in juvenile female rats, and oral DBP-treatment (250, 500 or

1000 mg/kg/d from weaning through adulthood) failed to accelerate vaginal opening, or to induce in constant estrus in intact female rats (Gray et al., 1999c).

Recent publications demonstrate that perinatal exposure to a number of phthalate esters alters development of the male reproductive tract in an antiandrogenic manner, causing underdevelopment and agenesis of the epididymis at relative low dosage levels. Arcardi et al. (1998) reported that administration of DEHP in the drinking water to the dam during pregnancy and lactation (estimated LOAEL of 3 mg/kg/d) produced testicular histopathological alterations in male rat offspring. Although DEHP is not an AR antagonist *in vitro* at concentrations up to 10 μM, DEHP inhibits fetal Leydig cell testosterone synthesis *in vivo* when orally administered to the dam at 0.75 g/kg/d starting at day 14 of pregnancy. As a consequence, fetal testosterone concentrations are reduced in males to female levels from day 17 of gestation to two days after birth. This reduction in testosterone levels results in a wide range of malformations of the androgen-dependent tissues in male rats including reduced AGD, retained nipples, hypospadias, cleft phallus, vaginal pouch, agenesis of the gubernacula cords and sex accessory tissues, underdevelopment of levator ani muscles, undescended testis, hemorrhagic testes, epididymal agenesis and testicular atrophy. Mylchreest et al. (1999) observed similar malformations in male rat progeny after prenatal oral exposure (day 10-22 of gestation) to DBP with effects occurring at dosage levels as low as 100 mg/kg/d. In a multigenerational assessment of the reproductive effects of DBP on the male and female parents and their progeny, daily oral administration of 500 mg/kg/d by gavage delayed puberty in male rats and reduced fertility in both male and female rats (Gray et al., 1999c) while 250 mg/kg/d induced reproductive tract malformations and reduced fecundity in the offspring. In addition, when dams were dosed by gavage with 500 mg DBP/kg in oil or an equimolar dose of DEHP (750 mg/kg/d) during sexual differentiation (GD14 - PND 4) the male offspring were profoundly malformed. More limited dosing in "pulses" during 4-day periods of gestation demonstrated that DBP at this dose was most effective on days 16-19 (Gray et al., 1999c).

Administration of DBP during the postnatal period (500 mg/kg/day for 14-34 days, beginning pnd 22-23 or 35-36) caused only a decrease in testis, epididymis, and/or seminal vesicle weight (Ashby and Lefevre, 2000).

In a recent investigation (Gray et al., 2000), examined several phthalate esters to determine if they also altered sexual differentiation in an antiandrogenic manner. The authors hypothesized that the phthalate esters that altered testes function in the pubertal male rat (Foster et al., 1980) would also alter testis function in the fetal male and produce malformations of androgen-dependent tissues. In this regard, benzyl butyl phthalate (BBP) and DEHP phthalate were expected to alter sexual differentiation, while dioctyl tere- (DOTP), diethyl (DEP) and dimethyl (DMP) phthalate would not. The authors also expected that the phthalate mixture, diisononyl phthalate (DINP), would be weakly active due to the presence of some phthalates with a 4-7 carbon side chain. DEHP, BBP, DINP, DEP, DMP, or DOTP were administered orally to the dam at 0.75 g/kg from gestational day 14 to postnatal day three. Male, but not female, pups from the DEHP and BBP groups displayed shortened AGDs (25%) and reduced testis weights (30%). As infants, males in the DEHP, BBP and DINP groups displayed female-like areolas/nipples (86%, 70% and 22% respectively) versus 0% in other groups and they displayed reproductive malformations. The percentages of males with malformations was 91%

for DEHP, 84% for BBP and 7.7% ($p < 0.04$) in the DINP group. These phthalate esters produced a wide range of malformations of the external genitalia, sex accessory glands, epididymides and testes. In the DINP group, 2/52 males (from 2/12 litters) displayed nipples, another male from one of the above litters displayed bilateral testicular atrophy and a fourth male in the DINP group, from a third litter, displayed unilateral epididymal agenesis with hypospermatogenesis and scrotal fluid-filled testis, devoid of spermatids.

In summary, DEHP, BBP, DBP and DINP all induce antiandrogenic effects on sexual differentiation. DEHP and BBP were of equivalent potency while DINP was about an order of magnitude less active.

Butyl benzyl phthalate (BBP), administered to male weanling rats (500 mg/kg/day for 14 days beginning on pnd 22-23, or 20 days beginning on pnd 35-36) caused increased liver weight, and increased combined kidney weight, but no effect on accessory sex organ weight (Ashby and Lefevre, 2000)

It is evident that the phthalate esters are high production volume toxic substances that have not been adequately tested for transgenerational effects. It also is clear that standard developmental toxicity studies are inadequate in their assessment of the effects of antiandrogens like the phthalates on the reproductive system (Ema et al.1993, 1994; Tyl et al., 1988). Developmental toxicity studies, conducted in several reputable laboratories using dosage levels well above those used herein, have failed to detect these malformations. There are many reasons for the insensitivity of the developmental toxicity test to detect reproductive tract malformations in the offspring. In studies conducted under regulatory test guidelines prior to 1998, exposure to the dams was on gestational day 6 to 15 in the rat, which is prior to the development of the fetal reproductive tract. In 1998, the period of exposure was extended to gestational day 19. However, this change did not resolve the major limitation of this protocol. Even when dosing is continued to day 19 of gestation, very few of the reproductive malformations cited above will be detected in a routine teratologic evaluation. Not only are some of these tissues very small and only detectable histologically, but also, the process of sexual differentiation continues through perinatal life in the rat and some reproductive tissues are not fully differentiated until puberty is complete. For these reasons, the reproductive system cannot be fully evaluated during perinatal life.

Linuron. Linuron is a urea-based herbicide with an acute oral LD50 for rats of 4000 mg/kg. Existing *in vitro* data demonstrate that linuron is a weak AR ligand (Cook et al., 1993; Waller et al., 1996a,b) with an EC50 between 64-100 μM. However, it has not been determined if linuron is an AR agonist or antagonist and the antiandrogenic potential of linuron has not been studied using sensitive *in vivo* assays at nontoxic dosage levels. Lambright et al. (2000) utilized a battery of *in vivo* and *in vitro* assays to assess whether linuron altered AR function. Linuron competed *in vitro* with an androgen for rat prostatic AR (EC50 = 100-300 μM) and human AR (hAR) in a COS cell whole cell binding assay (EC50 = 20 μM) and linuron inhibited dihydrotestosterone (DHT)-hAR induced gene expression in CV-1 and MDA-MB-453 cells (EC_{50} = 10 μM). Linuron-treatment (100 mg/kg/d oral for 7 days) reduced testosterone- and DHT-dependent tissue weights in the Hershberger assay (1953; using castrated, immature,

testosterone propionate-treated male rats) and linuron-treatment (100 mg/kg/d oral for 4 days) altered the expression of androgen-regulated genes in ventral prostate *in situ*.

The effects of linuron treatment *in vivo* are difficult, if not impossible, to detect in adult animals, but are quite apparent in the offspring when administered during gestation. In a modified multigenerational study, the only effects seen in P0 male rats when linuron was administered from weaning, through puberty, breeding and lactation at 0, 20 or 40 mg/kg/d by gavage in oil was a 2.5 day delay in PPS and a small reduction in seminal vesicle and cauda epididymal weights. Fertility and serum hormone levels were unaffected in the P0 generation at dosage levels up to 40 mg/kg/d (Gray et al., 1999c). In contrast, dramatic effects were seen in the F1 generation, including malformations and subfertility. The F1 pairs sired fewer pups under continuous breeding conditions (63 pups versus 104, mated continuously over 12 breeding cycles) and the F1 males had reduced testes and epididymal weights, and lower testes spermatid numbers. These developmental effects were surprising because it has been reported that when linuron was administered in the diet at concentrations up to 125 ppm over 3 generations reproductive malformations were not reported (Hodge et al., 1968) and Khera et al. (1978) reported that linuron was not teratogenic in the rat at dosage levels up to 100 mg/kg/d.

To resolve this apparent discrepancy, Gray et al. (1999c) administered linuron by gavage in oil at 100 mg/kg/d from days 14-18 of gestation. Anogenital distance (AGD) in male offspring, adjusted by analysis of covariance for body weight, was reduced by about 30% (pup weight was down 20%) and the incidence of areolas (with and without nipples) seen in the male offspring as infants was increased from 0% in controls to more than 44% in the linuron-treated males. Linuron treatment also induced epispadias in 1/13 males (partial hypospadias with the urethral opening half way down the phallus) and several androgen-dependent tissues were reduced in size in linuron-treated male rats, including the seminal vesicles, ventral prostate, levator ani/bulbocavernosus muscles, and epididymides. While the above effects are consistent with the action of a relatively weak AR antagonist, the high incidences of epididymal and testicular malformations (>50% of the linuron-treated males displaying agenesis or atrophy of one or both organs) were surprising. The epididymal malformations seen in treated male offspring included agenesis of the caput and/or corpus epididymides, while some testes were atrophic, fluid filled, and flaccid (Gray et al., 1999c; Lambright et al., 2000). These malformations also are produced at lower dosage levels. McIntyre et al. (1999, 2000) detected malformations in male rat offspring at dosage levels of linuron as low as 12.5 mg/kg/d (days 10-22 of gestation), the lowest dose examined. These data demonstrate that linuron is an AR antagonist both *in vivo* and *in vitro*. However, linuron produces a profile of malformations that differs from the standard AR antagonist, one that curiously resembles the effects seen with DBP or DEHP treatment (see below). It remains to be determined if linuron alters sexual differentiation by additional mechanisms of action in addition to AR antagonism or if tissue specific metabolites are formed.

Procymidone. Procymidone is a dicarboximide fungicide structurally related to vinclozolin. *In vitro*, procymidone inhibits DHT-induced transcriptional activation at 0.2 μM in CV-1 cells cotransfected with the human AR and a MMTV-luciferase reporter gene; at 10 μM DHT-induced transcriptional activity is completely inhibited (Ostby et al., 1999). Using a Chinese Hamster ovary (CHO) cell promoter interference assay, it was demonstrated that 1 μM

procymidone also blocked DHT-induced AR-DNA binding. The fact that these effects were seen at a concentration more than a 1000-fold below the Ki of the parent material for AR suggests that a procymidone metabolite(s) is the active antiandrogenic compound(s).

When administered by gavage at 100 mg/kg/d on gestational day 14 to day 3 after birth, procymidone reduces anogenital distance in male pups and induces retained nipples, hypospadias, cleft phallus, a vaginal pouch and reduced sex accessory gland size in male rat offspring (Gray and Kelce, 1996; Gray et al., 1999c). When administered at lower dosage levels using the same protocol (25, 50, 100, 200 mg/kg/d) effects were noted at all dosage levels (Ostby et al., 1999). Procymidone exposure reduced anogenital distance (at 25 mg/kg/d and above) and induced nipples (25 and above) and permanently reduced the size of several androgen-dependent tissues (levator ani and bulbocavernosus muscles (25 and above), prostate (50 and above), seminal vesicles (100 and above), Cowper's gland (100 and above), and glans penis (100 and above)) and induced malformations (hypospadias (50 and above), cleft phallus (50 and above), exposed os penis, vaginal pouch (50 and above) and ectopic, undescended testes (200)). Procymidone had a marked effect on the histology of the dorsolateral and ventral prostatic and seminal vesicular tissues (at 50 mg/kg/d and above). The effects consisted of fibrosis, cellular infiltration, and epithelial hyperplasia (Ostby et al., 1999). In contrast to the developmental effects, procymidone has little effect, if any, on the reproductive tract of the adult male rat (two weeks at dosage levels as high as 2000 ppm in the diet; Hosokowa et al., 1993).

Since the role of androgens in mammalian sexual differentiation is highly conserved, it is likely that humans would be adversely affected by vinclozolin or procymidone in a predictable manner if the human fetus was exposed to sufficient levels of the active metabolite(s) during critical stages of intrauterine life.

Chlozolinate and Iprodione. Chlozolinate and iprodione are dicarboximide fungicides, similar in structure to the antiandrogens vinclozolin and procymidone. However, when chlozolinate and iprodione were administered at 100 mg/kg/d from GD 14 to PND 3, male rat offspring were not demasculinized or feminized at this dosage level (Gray et al., 1999c).

Other antiandrogens. Male reproductive organ weight has been shown to be a sensitive indicator of anti-androgen activity in the peripubertal rodent for a variety of compounds. Ashby and Lefevre (2000) demonstrated that cyproterone and flutamide, each administered at 25 mg/kg for 14 consecutive days beginning on pnd 22-23 or 35-36, resulted in decrease weight in the testes, epididymides, seminal vesicles, and/or prostate, in the absence of a decrease in body weight. Another antiandrogen, 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene, increased liver weight or decreased body weight, but had no effect on male reproductive organ weight.

3.5.4 Metabolic Inhibitors

Several fungicides inhibit membrane synthesis and growth by inhibiting specific cytochrome P450 enzymes, especially 14α demethylation of lanosterol in the sterol pathways. The process of steroidogenesis is sufficiently conserved across taxa that these chemicals also inhibit mammalian steroidogenesis. In general, however, at relatively high concentrations these

fungicides are nonspecific inhibitors of CYP450 enzymes. Hence, effects in vertebrates may not be limited to the reproductive system and include adrenal and liver steroid metabolism and ecdysteroid synthesis in invertebrates.

Ketaconazole. Goldman et al. (1976) produced male pseudohermaphroditism in rats with inhibitors of steroid 17α-hydroxylase and C17-20 lyase. Developmental alterations also can be obtained from *in utero* treatment with drugs that inhibit 5α-reductase, which is not a P450 enzyme, blocking the conversion of testosterone to dihydrotestosterone (Imperato-McGinley et al., 1992; Wier et al., 1990). The antifungal imidazole derivative, ketoconazole, inhibits various enzymes which belong to the cytochrome-P450-dependent mono-oxygenases in rodents and humans, which act to catalyze side chain cleavage of cholesterol, 11β-hydroxylase in the adrenals, and 17α-hydroxylase and C17-20 lyase in rat and human testes. For example, human testicular mono-oxygenase activities *in vitro* are reduced by 50% by 3.1 μM ketoconazole. Schurmeyer and Nieschlag (1984) demonstrated that ketoconazole and other imidazole fungicides inhibited T production in males, while Pepper et al. (1990) reported that ketoconazole was useful in the treatment of ovarian hyperandrogenism in women. Ketoconazole also has been shown to alter hepatic testosterone metabolism (Wilson and LeBlanc, 2000). Four hours after male CD-1 mice were orally treated with ketoconazole from 0 to 160 mg/kg, serum testosterone levels, gonadal testosterone production and hepatic testosterone hydroxylase activity were decreased in a dose-related manner.

Administration of ketoconazole by gavage from day 14 of pregnancy in the rat at 100 mg/kg/d leads to reduced maternal weight gain and whole litter loss within a few days of the initiation of treatment (Gray et al., 1999c). These effects are consistent with the ability of this fungicide to inhibit progesterone synthesis. When the dosage level of ketoconazole was lowered to 0, 12.5, 25, or 50 mg/kg/d from day 14 of gestation to postnatal day 3, treatment delayed the onset of parturition by as much as three days and reduced the numbers of live pups at all but the lowest dosage level. Surviving male pups in the 12.5 and 25 mg/kg/d dose groups (there was only 1 pup at 50) did not display any indication of being demasculinized or feminized (Gray et al., 1999c). Administration of ketaconazole to male rat weanlings (15 mg/kg/day for 14 consecutive days, beginning on pnd 35-36) caused decreased epididimyde weight, whereas at 25 mg/kg/day, seminal vesicle weight and prostate weight were decreased (Ashby and Lefevre, 2000).

Fenarimol. Aromatase inhibitors prevent the conversion of androgens to estrogens. This P-450 enzyme is highly conserved in a wide variety of tissues and many species, but the overall homology of the gene with other cytochrome P-450s is only about 30%. Hence, this enzyme is considered to be in a separate gene family within the overall superfamily. As a consequence of the lack of sequence homology with other P450 enzymes in the steroid pathway, inhibitors of aromatase can display more specific activities than drugs like ketoconazole. In this regard, aromatase inhibitors have significant clinical uses and often present fewer untoward effects on other endocrine organs.

Some fungicides like fenarimol inhibit the P450 enzyme aromatase, preventing the conversion of androgens to estrogens (Hirsch et al., 1986, 1987). In a series of experiments in rats, Hirsch et al. (1986) evaluated the effect of feniramol exposure through the diet on two

generations exposed to 50, 130, and 350 ppm fenarimol. F0 males exhibited a dose- and time-dependent decrease in fertile matings. This effect was more pronounced in the F1 males, in the absence of reproductive tract malformations. A crossover mating trial in which 35 mg/kg/day fenarimol was administered by gavage (equivalent to the exposure of the 350 ppm treatment group) revealed that although treated males were anatomically normal, with normal spermatogenesis, they produced fewer fertile matings. The absence of vaginal sperm in the unsuccessful matings suggested that, since the treated males appeared to have normal spermatogenesis, and treated females were cycling normally, fenarimol was acting centrally to disrupt male mating behavior, which is dependent on steroid activity in the CNS during perinatal development. Additional studies revealed that fenarimol was concentrated in the milk, and that uptake into the neonatal hypothalamus was more rapid, and reached higher levels than in the brain as a whole. In another study, fenarimol was administered daily by gavage from weaning, through puberty, mating, gestation and lactation to the P0 generation (at 0, 17.5, 35, or 70 mg/kg/d) while the F1 offspring were only exposed indirectly through the placenta and milk (Hirsch et al., 1987). When fenarimol was administered continuously from weaning, treated male rats displayed altered mating behavior (failure to mount a sexually receptive female). As this behavior is dependent upon the conversion of testosterone to estradiol in the brain of the male rat, this effect likely resulted from an inhibition in the production of estrogens in the brain (Gray and Ostby, 1998). Serum hormone levels were unaffected by fenarimol treatment but male rats from the highest dosage group displayed reduced sex accessory gland weights and increased liver size, effects that could indicate that fenarimol was inhibiting P450 enzymes in the testis and liver. This chemical also increased ovarian weight and caused delayed parturition in female rats because of the critical role of estrogens near term in the induction of labor in this species. Although fertility in the P0 generation was reduced by fenarimol treatment, the F1 generation, exposed indirectly only during pregnancy and during lactation, was unaffected.

3.5.5 Other

In an evaluation of non-receptor mediated endocrine active compounds, 6-propylthiouracil (PTU) (a potent thyroid modulator; 240 mg/kg/day), phenobarbital (PB) (a weak thyroid modulator, 50 or 100 mg/kg/day), haloperidol (HALO) (adopamine antagonist, 2 or 4 mg/kg/day), or bromocryptine (BRC) (a dopamine agonist, 10 or 50 mg/kg/day) were administered by gavage to weanling CD rats on pnd 21-51 (Marty et al., 2001). PTU caused delayed PPS and growth; decreased testis and epididymis weights, decreased relative thyroid weight, decreased serum T4, DHT, and testosterone levels, increased TSH levels and relative thyroid weight, and altered thyroid histology indicative of increased TSH secretion. PB treatment resulted in decreased absolute testis, epididymis, prostate and seminal vesicle weights at high dose; increased relative thyroid weight, and decreased T4. HALO caused increased relative thyroid weight and decreased T4, and BRC treatment resulted in delayed PPS and growth at high the dose; and decreased absolute prostate and seminal vesicle weight. This study of the effect of thyroid antagonists and dopamine agonists indicates that PPS, thyroid weight, T4 and TSH secretion and reproductive organ weight are parameters that are sensitive enough to identify and distinguish between potent and weak thyroid antagonists, and dopamine agonists. In another study conducted by Aguilar et al., (1988), male rats were grafted on pnd 21 with pituitary glands from either pnd 21 rats or pnd 90 rats, and then treated with bromocriptine. Evaluation of acquisition of puberty in the absence of bromocryptine indicated that all the rats

with grafts experienced precocious puberty (~36.5 days vs. 40 days for controls) and increased prolactin concentration. Bromocryptine administration reduced prolactin levels in both groups, and blocked the occurrence of precocious puberty in rats give pnd 90 pituitaries, but not in those given pnd 21 pituitaries. These results suggest that precocious puberty induced by the "adult" pituitaries was prolactin dependent, whereas that induced by the "young" pituitary occurred by another mechanism. The validity of using these endpoints to detect dopamine agonists is still uncertain.

4.0 CANDIDATE PROTOCOLS

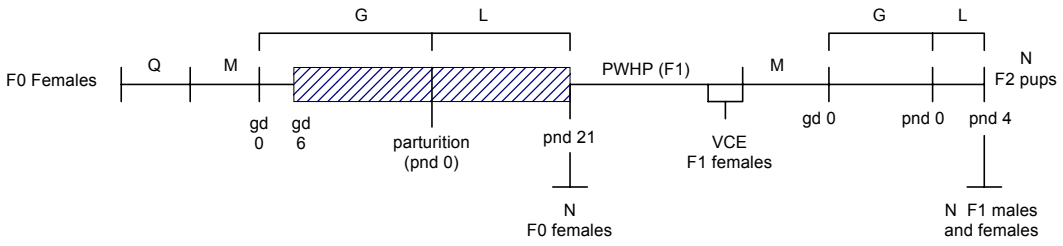
The EDSTAC recommended that a mammalian reproductive toxicity protocol be developed for use in evaluating possible endocrine-disruption activities of compounds after *in utero*/lactational exposure, to be used as screening study. The protocols were to be designed to assess the postnatal developmental and reproductive consequences of *in utero* and lactational exposures to chemical substances or mixtures with possible estrogen, androgen, and/or thyroid activities. Three candidate study designs are presented below. Candidate Protocol A is the FDA Segment III, Perinatal/Postnatal Study. It is presented as an example of a *in utero*/lactational exposure study design not specifically targeted toward identifying endocrine disruption. Candidate Protocol B and C are study designs that were developed in response to the EDSTAC recommendation and have not been tested, although they include aspects of study designs that have been used previously.

4.1 CANDIDATE ASSAY A – FDA SEGMENT III PERINATAL/POSTNATAL STUDY

4.1.1 System Model – *in Vivo* Pre- and Postnatal Exposure in Rodents

The FDA Segment III or FDA Perinatal/Postnatal Protocol is a current guideline study for food additives and pharmaceutical agents that employs *in utero*/lactation exposure and postnatal evaluation. It is presented here for comparison.

4.1.2 Flow Diagram of the FDA Segment III Study (Protocol A)



Key:

Q = Quarantine (one week)
M = Mating (approximately one week for F0 females, two weeks for F1 animals)
G = Gestation (approximately 21-23 days)
gd = Gestational day
L = Lactation (21 days)
pnd = Postnatal day
N = Necropsy
PWHP = Postwean holding period (minimum 49 days, so F1 offspring are at least 70 days old at end of this period)
VCE = Vaginal cyclicity evaluation of F1 females for 21 days immediately prior to mating
Gavage dosing of F0 females, gd 6 through pnd 21

4.1.3 Description of the FDA Segment III Study

4.1.3.1 Species Tested, Including Specifics on Strain or Stock, Sex, Age Range, Diet, Etc.

This protocol has been conducted in rats. Due to the volume of historical reproductive and developmental data describing Sprague-Dawley-derived rats, this would be the most obvious choice of a test animal. Female age at mating should be 10-12 weeks of age. Standard laboratory diet should be used. The method of euthanasia for adult or weanling scheduled or unscheduled terminations will be by CO2 asphyxiation, unless an endpoint to be selected is affected by that form of asphyxiation. Decapitation or cervical dislocation are inappropriate for use on adults and weanlings based on the most recent NIH and other government guidelines for the care and use of animals. Termination of the pnd 4 culled pups or termination of young moribund pups will be by decapitation, since a very young animal is resistant to termination by asphyxiation; decapitation of young rats, mice, and rabbits is the preferred method of termination according to the NIH and other government regulations for the care and use of animals.

4.1.3.2 Chemical/product Evaluated, Including Chemical/product Name (If Mixture, Provide

Information on All Components), Cas Registry Number, Chemical/product Class, Physical/chemical Characteristics Directly Related to Test Performance (E.g., Water and Lipid Solubility, Ph, Pka, Etc), Stability of Test Material in Test Medium, Purity, Supplier/source. Not applicable.

4.1.3.3 Route of Administration.

Gavage is the most appropriate route of administration since it delivers a precise dose and prevents direct exposure of the pups to the test compound.

4.1.3.4 Doses Used (Including Vehicle Controls).

Doses used in this protocol generally allow for 90% maternal survival at the high dose, in the absence of a more specific toxicity.

4.1.3.5 Duration of Exposure.

Maternal animals are directly dosed (i.e., gavage) from gd 15 through pnd 21 (weaning). This period of exposure encompasses the period of late *in utero* development (after organogenesis) through the lactational period. Offspring would potentially be exposed to the test compound *in utero* after organogenesis and through the mother's milk during lactation.

4.1.3.6 Appropriate Vehicle, Negative And/or Positive Controls; Basis for Selection.

An appropriate vehicle would be one that has no endocrine activity or other overt toxicity and that provides a stable dose formulation that can be accurately analyzed for test compound concentration.

4.1.3.7 Endpoints/Data Recorded.

Individual Maternal Data for the F0 and F1 Generations:

a. Identification number
b. Age at beginning of study
c. Age at death and manner of death
d. Weekly body weights prior to mating (F1 only) and gestational and lactational body weights
e. Gestational and lactational feed consumption (F0 only)
f. Male rat (by identification number) used for mating
g. Gestation length in days
h. Total number of offspring per litter
i. Number and percent of live and dead offspring
j. General condition of offspring and mother through weaning (F0 dams) or pnd 4 (F1 dams)

k. Prebreed estrous cyclicity (F1 only)
l. Precoital interval (F1 only)
m. Gross necropsy
n. Number of uterine implantation (nidation) scars at necropsy

Summary of Maternal Data for the F0 and F1 Generations:

a. Mean periodic maternal body weights and weight gains
b. Feed consumption during gestational and lactational periods (F0 only)
c. Survival indices
d. Mean litter size
e. Mean number of live and dead offspring
f. Number and percent of mothers showing treatment-related behavioral abnormalities in nesting and nursing

- Mating index (%) = $\frac{No.\ females\ sperm\ positive}{No.\ females\ paired} x100$
- Fertility index (%) = $\frac{No.\ females\ pregnant}{No.\ females\ sperm\ positive} x100$
- Prenatal (postimplantation) loss (%) =

$$\frac{No.implantation\ scars - No.\ pups\ born\ alive}{No.implantation\ scars} x100$$

- Prebreed estrous cyclicity (F1 only)
- Precoital interval (F1 only)
- Gross necropsy

Individual Paternal Data for the F1 Generation:

- Identification number
- Age at beginning of study
- Age at death and manner of death
- Weekly body weights
- Gross necropsy

Summary of Paternal Data for the F1 Generation:

- Mean periodic paternal body weights and weight gains
- Mating index (%) = $\frac{No.males\ impregnating\ females}{No.males\ paired} x100$
- Fertility index (%) = $\frac{No.males\ siring\ litters}{No.males\ impregnating\ females} x100$

- Pregnancy index (%) = $\frac{No.\ pregnant\ females}{No.\ males\ impregnating\ females} x 100$
- Gross necropsy

Summary of Litter Data for the F1 and F2 Generations:

- Total litter size
- Number and percent of stillborn
- Number and percent of live births
- Periodic viability counts
- Periodic body weights by sex per litter from birth to weaning for F1 pups and from birth to pnd 4 for F2 pups (Taken by individual pup)
- Sex ratio (% males per litter)
- Indices:

  F1 and F2 generations:

  Gestational index = $\frac{Number\ of\ females\ with\ live\ litters}{Number\ of\ females\ pregnant}$

  Live birth index = $\frac{Number\ of\ live\ pups\ at\ birth}{Total\ number\ of\ pups\ born}$

  4-day survival index = $\frac{Number\ of\ pups\ surviving\ 4\ days}{Total\ number\ of\ live\ pups\ at\ birth}$

  F1 generation only:

  7-day survival index = $\frac{Number\ of\ pups\ surviving\ 7\ days}{Total\ number\ of\ live\ pups\ at\ 4\ days}$

  14-day survival index = $\frac{Number\ of\ pups\ surviving\ 14\ days}{Total\ number\ of\ live\ pups\ at\ 7\ days}$

  21-day survival index = $\frac{Number\ of\ pups\ surviving\ 21\ days}{Total\ number\ of\ live\ pups\ at\ 14\ days}$

  Lactation index = $\frac{Number\ of\ pups\ surviving\ 21\ days}{Total\ number\ of\ live\ pups\ at\ 4\ days}$

g. Developmental landmarks (F1 only)
   - pinna detachment (prewean)
   - eye opening (prewean)
   - vaginal patency (postwean)
   - preputial separation (postwean)

h. Functional assessments (F1 only)
   - eye examination (postwean)
   - auditory function (postwean)
   - motor activity (postwean)

learning and memory (postwean)

4.1.3.8 Relevance of Data Collected and Associated Endpoints.

The data collected in the standard Segment III study sheds light on general viability, growth, and development of animals after late gestational and lactational exposure. However, with the exception of the determination of sex ratio on pnd 0 and the examination and histopathology of the reproductive organs, it is not specifically targeted to detect endocrine disrupting compounds. In addition, the exposure during late gestation precludes evaluation of perturbations of the endocrine system which occur early in gestation, including genesis of the reproductive organs.

4.1.3.9 Known False Negatives, Known False Positives.

May miss many late effects on the reproductive system.

4.1.3.10 Sensitivity of Assay, Lowest Level of Detection.

Due to the late gestational exposure and the lack of hormone assays, this protocol, as it has been described, would not provide a sensitive evaluation of endocrine disrupting activity.

4.1.4 Strengths of the Segment III Study

4.1.4.1 Overall Protocol Design.

The overall study design is appropriate, as has been shown by its use in government-sponsored testing.

4.1.4.2 Route of Administration/exposure.

A frequently used route of administration of the test compound is oral, by gavage. The oral route of administration is appropriate, mimicking a likely route of exposure to endocrine-disrupting compounds that is anticipated to occur in humans. The use of gavage is important during the lactational period, since it prevents direct exposure of the pups to the test compound, as might happen with dosed feed or water.

4.1.4.3 Dosing Period/duration.

The dosing period in the FDA Segment III study is from gd 15 to pnd 21 (at weaning). This dosing period encompasses late gestational development of the reproductive tract, after organogenesis is complete, and early postnatal sexual differentiation. The FDA Segment III was designed to be preformed with a Segment I study (dosing prior to mating, during mating, and until implantation on gd 6), and with a Segment II study (dosing to the pregnant female from implantation on gd 6 to the end of major organogenesis on gd 15). When all three are done, the entire process of development is evaluated from mating of F0 parents through the reproduction of F1 offspring to make the F2 generation. However, the segment III study, as initially designed

is the only one that looks at long term postnatal consequences of late in utero/lactational exposure, and it did not in its initial design of the Segment III study start exposures until after major organogenesis is complete. The International Commission of Harmonization (ICH) deliberations resulted in modification of the Segment III study to start exposures to the dam on gd 6. The rest of the protocol was left intact, and it now is a very powerful assessment of postnatal consequences of in utero and lactational exposure from implantation of conceptuses into the uterine lining on gd 6. The authors therefore have presented the Segment III protocol as modified by ICH. Please note that it is now similar to Protocol B.

4.1.4.4 Measurements/endpoints.

Basic endpoints of survival and growth are included in this protocol. As a means of external sexing, AGD is the one endpoint specific to endocrine disruption that is included other than gross necropsy and examination of the reproductive tract on pnd 14.

4.1.5 Weaknesses of the Segment III Study

4.1.5.1 Overall Protocol Design.

Evaluation of the immature reproductive system in inadequate.

4.1.5.2 Route of Administration/Exposure.

No weaknesses found.

4.1.5.3 Dosing Period/duration.

The dosing period in the original Segment III study design encompasses only the late gestation and lactational period of sexual differentiation. This protocol would not detect disruptions in the ontogeny of the reproductive tract that occurs prior to the fetal period during organogenesis. The ICH also recognized this shortcoming and proposed starting exposures to the F0 maternal animal on gd 6 at the time of implantation of the conceptuses (see Strengths, above).

4.1.5.4 Measurements/endpoints.

With the exception of AGD and gross necropsy of the reproductive tract on pnd 14, no specific endpoints for endocrine disruption are included. These could be added to the assay, however, if necessary.

4.1.6 Test Method Performance - Statistical Power of Approach

The statistical power of the analysis is adequate.

4.1.7 Test Method Reliability - Assurance of *in Vivo* Biological Test Vigor, Sensitivity, and Reproducibility

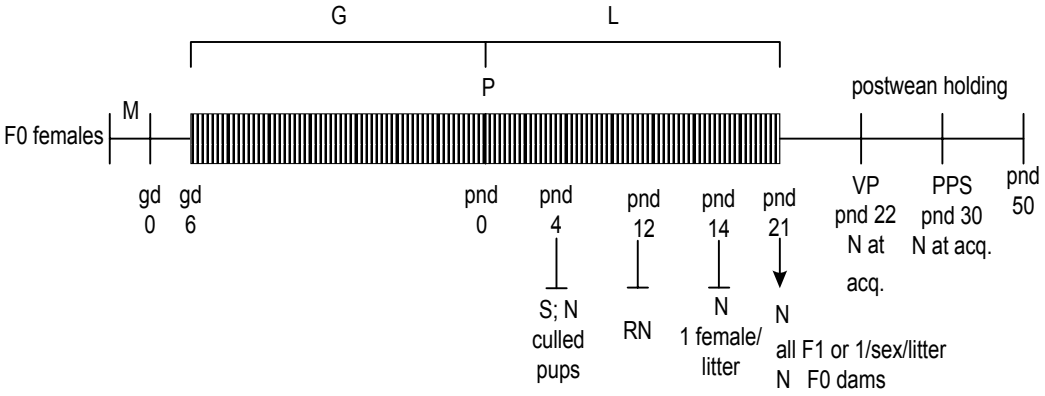
No weaknesses found.

4.2 CANDIDATE ASSAY B – PROTOCOL FOR A POSSIBLE *IN UTERO*/LACTATIONAL EXPOSURE ASSAY

4.2.1 System Model – *in Vivo in Utero*/lactational Exposure in Rodents

In response to the recommendation of the EDSTAC, an *in utero* developmental screening assay was developed by Drs. L. E. Gray and R. W. Tyl (members of the EDSTAC Screening and Testing Work Group) and is described below (see also Zenick et al., 1993)

4.2.2 Flow Diagram of the Possible *in Utero* Developmental Screening Assay (Protocol B)



Key:

Direct exposure of F0 dams by gavage once daily on gd 6 through pnd 10 or 21
M = mating

G = gestation

gd = gestational day

P = parturition

pnd = postnatal day

L = lactation

S = standardize litters on pnd 4 to 10

RN = examination of male F1 pups on pnd 12 for retained nipples

N = necropsy

VP = acquisition of vaginal patency in F1 females beginning on pnd 22

PPS = acquisition of preputial separation in F1 males beginning on pnd 30

acq = acquisition

4.2.3 Description of Protocol B

4.2.3.1 Species Tested, Including Specifics on Strain or Stock, Sex, Age Range, Diet, Etc.

Like the Segment III study, the Gray and Tyl study protocol is designed to be conducted in rats. Due to the volume of historical reproductive and developmental data describing Sprague-Dawley-derived rats, this would be the most obvious choice of a test animal. Female age at mating should be 10-12 weeks of age. Standard laboratory diet should be used.

4.2.3.2 Chemical/product Evaluated, Including Chemical/product Name (If Mixture, Provide Information on All Components), Cas Registry Number, Chemical/product Class, Physical/chemical Characteristics Directly Related to Test Performance (E.g., Water and Lipid Solubility, Ph, Pka, Etc), Stability of Test Material in Test Medium, Purity, Supplier/source.

This protocol has not been tested.

4.2.3.3 Route of Administration.

Gavage is the most likely route of administration. As with Protocol C, the route of administration for testing should mimic the expected route of exposure of humans and wildlife to the putative endocrine disruptor.

4.2.3.4 Doses Used (Including Vehicle Controls).

The doses chosen should be based on information obtained through the other data. The EDSTAC recommended that doses be relevant to human/wildlife exposure levels for testing. At least 2 treated groups plus vehicle control should be used. The maximum number of doses would depend on cost and logistical limitation of the performing laboratory.

4.2.3.5 Duration of Exposure.

Direct exposure of the maternal animals (i.e., by avage) will be from gd 6 through pnd 21 (i.e., weaning of the pups). This period of exposure encompasses the entire period of *in utero* development (after implantation) through the lactational period. Offspring would potentially be exposed to the test compound *in utero*, and through the mother's milk during lactation.

4.2.3.6 Appropriate Vehicle, Negative And/or Positive Controls; Basis for Selection.

An appropriate vehicle would be one that has no endocrine activity or other overt toxicity, and that provides a stable dose formulation that can be accurately analyzed for test compound concentration. It is advisable that both positive and negative controls for estrogenic, androgenic, and thyroidogenic activity be used during the validation process.

4.2.3.7 Endpoints Measured.

Maternal

- In-life: body weights, feed consumption, clinical observations
- Necropsy: body weight, liver weight, thyroid weight, postimplantation prenatal loss, blood T4/TSH; possible thyroid histopathology

Offspring

- Apparent sex ratio on pnd 0; with body weight from birth until weaning
- Postnatal survival and development
- Reproductive tract anomalies and uterine weight on pnd 4
- Nipple retention

- Female reproductive tract anomalies and uterine weight, possible uterine histopathology on pnd 14
- Male and female reproductive tract anomalies, weight of testis, epididymides, uterus, and ovaries; examine for precocious puberty (acquisition of vaginal patency) and vaginal threads, T4/TSH (possible E2 in females and T in males) on pnd 21
- Postwean weight weekly, clinical observations daily, acquisition of VP (and vaginal threads) for females and PPS for males
- On day of acquisition of VP and PPS, weigh animals and necropsy, examine as on pnd 21, weigh and retain thyroid, testes, epididymides, ovaries, and uterus; T4/TSH, E2, and T

4.2.3.8 Data Collected.

Maternal

- In-life: body weights, feed consumption, clinical observations
- Necropsy: body weight, liver weight, thyroid weight, uterine implantation sites counted (for postimplantation prenatal loss), blood samples for T4/TSH; thyroid retained in fixative for possible thyroid histopathology

Offspring

- Apparent sex ratio (by measured anogenital distance on pnd 0) with body weight on pnd 0 (at birth), and on pnd 4, 7, 14, and 21 (anogenital distance not measured)
- Postnatal survival and development
- At necropsy on pnd 4: reproductive tract anomalies (e.g., hypospadias), missing, small, or ectopic testes or ovaries, missing or small epididymides, missing seminal vesicles or oviducts, presence of Wolffian ducts or their derivatives (epididymides and seminal vesicles) in females, presence of Müllerian ducts or their derivatives (oviducts) in males, weigh uterus
- On pnd 10-12, examine males for retained nipples
- On pnd 14, necropsy females (one/litter), weigh uterus (possible histopathology to measure uterine gland number and luminal epithelial cell height), examine reproductive system for anomalies
- On pnd 21, necropsy one/sex/litter or all remaining pups, examine males for reproductive tract anomalies, weigh testes and epididymides, examine females for reproductive tract anomalies, weigh uterus and ovaries, examine for precocious puberty (acquisition of vaginal patency) and vaginal threads, take blood samples for T4/TSH (E2 in females? T in males?)
- If pups are retained postwean, weight weekly, clinical observations daily, evaluate for acquisition of VP (and vaginal threads) for females starting on pnd 22, evaluate for acquisition of PPS for males starting on pnd 30
- On day of acquisition of VP and PPS, weigh animals and necropsy, examine as on pnd 21, also weigh and retain thyroid, testes, epididymides, ovaries, and uterus; take blood samples for T4/TSH, E2, and T

4.2.3.9 Relevance of Data Collected and Associated Endpoints.

Interpretation of Endpoint Changes

- If anogenital distance changes (covary by body weight for statistical analysis):
  - increased in females from androgen
  - decreased in males from estrogen or anti-androgen
- If uterine weight changes:
  - increased precociously by estrogen
- If there are male reproductive tract anomalies: from anti-androgens or possibly estrogens (feminization)
- If there are female reproductive anomalies: from androgens, estrogens, or possibly anti-estrogens
- If changes in T4/TSH, thyroid weight (histopathology): from thyroid or anti-thyroid activity
- If change in age at acquisition of VP/PPS: accelerated VP from estrogens, delayed VP from anti-estrogens/androgens, accelerated PPS from androgen, delayed PPS from anti-androgen (covary age at VP or PPS by body weight at acquisition for statistical analysis)

4.2.3.10 Known False Negatives, Known False Positives.

This protocol has not been tested.

4.2.3.11 Sensitivity of Assay, Lowest Level of Detection.

This protocol has not been tested. However, serum estradiol, thyroxine, and TSH may provide a sensitive measure of perturbation of the endocrine system.

4.2.3.12 Statistical or Non-statistical Methods Used for Analysis.

The unit of comparison is the female, litter, or weanling. Group size allows use of parametric analysis to evaluate dose effect on selected measures using analysis of variance. When a significant ($p<0.05$) main effect for dose occurs, pairwise comparison of the control group with any treated group is conducted. Nominal scale measures are evaluated by the chi square test for dose effect, followed by pairwise comparison. Dose-related trends are also determined. See Section 6.8.

4.2.3.13 Decision Criteria Used to Classify a Test Chemical.

An endocrine sensitive parameter with a statistically significant ($p<0.05$) dose effect and a significant pairwise comparison between one dose level and the control group, in the presence of a significant dose-related trend, would be the strongest indication of test chemical effect at that dose level. However, observational data must also be taken into account in order to determine whether the numerical data is a reasonable reflection of the biological results.

4.2.4 Strengths of the Protocol B Study Design

4.2.4.1 Overall Protocol Design.

Protocol B is well-designed. The protocol provides for enough animals to evaluate each endpoint. The endpoints are logical, appropriate, and can be evaluated within the protocol design in a manner that does not disrupt the study conduct.

4.2.4.2 Route of Administration/exposure.

The proposed route of administration of the test compound is oral. The oral route of administration is appropriate, mimicking a likely route of exposure to endocrine-disrupting compounds that is anticipated to occur in humans. The use of gavage is important during the lactational period, since it prevents direct exposure of the pups to the test compound, as might happen with dosed feed or water.

4.2.4.3 Dosing Period/duration.

Exposure is to be to the pregnant dams on gd 6 through pnd 21 (weaning). The period of administration is appropriate in that it encompasses the critical *in utero* and lactational periods defined by other studies that have identified endocrine-disrupting compounds.

4.2.4.4 Measurements/endpoints.

This assay includes the following endpoints/measurements: AGD; E2; uterine histology; T4; TSH; thyroid histology; organ weights; vaginal cytology; vaginal patency; PPS; retained nipples; and male and female reproductive histology. These endpoints have been shown to be appropriate and sensitive to endocrine-disrupting activities in the rodent in previous studies.

4.2.5 Weaknesses of the Protocol B Assay

4.2.5.1

Overall Protocol Design. No overt weaknesses. However, the design may be too ambitious to serve as a screening study.

4.2.5.2 Route of Administration/Exposure.

No weaknesses found.

4.2.5.3 Dosing Period/duration.

No weaknesses found.

4.2.5.4 Measurements/endpoints.

No weaknesses found.

4.2.6 Test Method Performance - Statistical Power of Approach

The statistical power of the approach is adequate.

4.2.7 Test Method Reliability - Assurance of *in Vivo* Biological Test Vigor, Sensitivity, and Reproducibility

The endpoints proposed in this protocol have been shown to be sensitive and reproducible. It is likely that use of this protocol will provide a database of information that will be comparable across studies and across different compounds. Additional endpoints sensitive to changes in testosterone could be added.

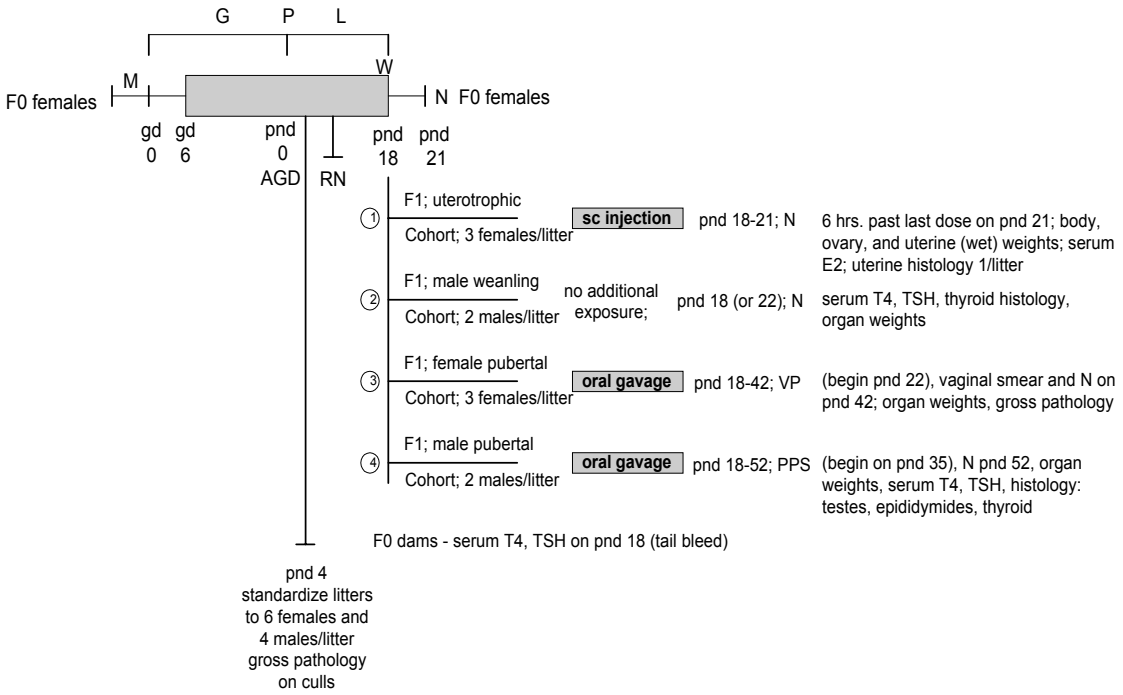
4.3 CANDIDATE PROTOCOL C – DEVELOPMENTAL TOXICITY PROTOCOL FOR EVALUATION OF ENDOCRINE DISRUPTION

In response to the recommendation of the EDSTAC, Dr. Robert A. Kavlock (NHEERL, EPA) convened a meeting on May 7, 1999, to develop a protocol to detect effects mediated by alterations in the estrogen, androgen, and/or thyroid signaling pathways as a consequence of exposure of the pre- and postnatal developing organism. It was intended to replace a number of EDSTAC Tier 1 screening assays. The attendees were Dr. Robert Chapin (NIEHS), Dr. Ralph Cooper (EPA), Dr. George Daston (Procter and Gamble), Dr. Paul Foster (CIIT), Dr. L. Earl Gray, Jr. (EPA), Dr. Robert Kavlock (EPA), Dr. Daniel Sheehan (NCTR), Dr. Dana Shuey (DuPont), and Dr. Rochelle Tyl (RTI). The protocol is described below.

4.3.1 System Model – *in Vivo in Utero*/lactational Exposure in Rodents

Dr. Robert Kavlock (NHEERL, EPA) convened a meeting on May 7, 1999, to develop a protocol to detect effects mediated by alterations in the estrogen, androgen, and/or thyroid signaling pathways as a consequence of exposure of the pre- and postnatal developing organism. It was intended to replace a number of EDSTAC Tier 1 screening assays.

4.3.2 Flow Diagram of the *in Utero*/lactational Screening Assay (Protocol C)



Key:

M = mating	RN = examination for retained nipples in F1 males on pnd 12-13
G = gestation	W = wean (pnd 18)
gd = gestational day	N = necropsy
P = parturition	VP = acquisition of vaginal patency (females)
pnd = postnatal day	PPS = acquisition of preputial separation (males)
AGD = anogenital distance on pnd 0	E2 = 17ß-estradiol
L = lactation	direct exposure

4.3.3 Description of the *in Utero*/lactational Screening Assay

4.3.3.1 Species Tested, Including Specifics on Strain or Stock, Sex, Age Range, Diet, Etc. This study protocol is designed to be conducted in rats. Due to the volume of historical reproductive and developmental data describing Sprague-Dawley-derived rats, this would be the most obvious choice of a test animal. Female age at mating should be 10-12 weeks of age. Standard laboratory diet should be used.

4.3.3.2 Chemical/product Evaluated, Including Chemical/product Name (If Mixture, Provide Information on All Components), Cas Registry Number, Chemical/product Class, Physical/chemical Characteristics Directly Related to Test Performance (E.g., Water and Lipid Solubility, Ph, Pka, Etc), Stability of Test Material in Test Medium, Purity, Supplier/source. This protocol has not been tested with a specific chemical.

4.3.3.3 Route of Administration. The F0 maternal animals will be administered test compound by gavage that optimizes exposure accuracy, since it is the goal of the screening study to define the intrinsic activity of the test compound on endocrine-dependent processes. Exposure to the dams ceases at weaning of the F1 offspring on pnd 18. The F1 offspring are then divided into two female cohorts and two male cohorts for post-weaning evaluation. Cohort 1 is the female uterotrophic assay with administration of the test material by subcutaneous (sc) injection on pnd 18-21. The selection of sc injection os the route was done to evaluate chemicals that are not active by the oral route, or that are rapidly metabolized if administered orally; it is also the standard route of administration for many uterotrophic assays. Cohort 3 (female) and cohort 4 (male) are basically pubertal assays with administration of the test material by oral gavage from pnd 18 (at weaning) through pnd 42 (females) and pnd 52 (males). Cohort 2 is a male weanling evaluation with immediate necropsy and evaluation of T4, TSH, and thyroid histology.

4.3.3.4 Doses Used (Including Vehicle Controls). The doses chosen should be based on information obtained through the other studies, if available. The screening study should be conducted with the highest dose at the MTD.

4.3.3.5 Duration of Exposure. Direct exposure of the maternal animals (i.e., by gavage) will be from gestational day (gd) 6 through postnatal day (pnd) 18 (i.e., weaning of the pups). This period of exposure encompasses the entire period of *in utero* development (after implantation) through the lactational period. Offspring would potentially be exposed to the test compound *in utero*, and through the mother's milk during lactation. F1 animals would be exposed directly from pnd 18 up to pnd 52.

4.3.3.6 Appropriate Vehicle, Negative And/or Positive Controls; Basis for Selection. An appropriate vehicle would be one that has no endocrine activity or other overt toxicity and that provides a stable dose formulation that can be accurately analyzed for test compound concentration. Distilled water or deionized water would be selected for water-soluble compounds. Corn oil would be the choice for compounds that are not water soluble. It is advisable that both positive and negative controls for estrogenic, androgenic, and thyroidogenic activity be used during the validation process.

4.3.3.7 Endpoints Measured. The endpoints measured include the following:

Maternal

- In-life: body weights, feed consumption, clinical observations
- Necropsy on pnd 18: body weight, liver weight, thyroid weight, uterine implantation sites counted (for postimplantation prenatal loss), T4/TSH; possible thyroid histopathology

Offspring

- Apparent sex ratio on pnd 0
- Body weight on pnd 0 (at birth), and on pnd 4, 7, 14, 18, and 21, 22, 42, or 52
- Postnatal survival and development
- Gross reproductive tract anomalies on pnd 4
- Retained nipples on pnd 12-13 males
- Uterotrophic effects on females dosed from pnd 18-21
- Male reproductive tract anomalies on pnd 18 or 22; T4, TSH, thyroid histology
- Acquisition of vaginal patency in females dosed orally from pnd 18 to 42
- Acquisition of preputial separation (PPS) in males dosed from pnd 18 to 52; T4, TSH; histology on reproductive organs and thyroid on pnd 52.

4.3.3.8 Data Collected.

Maternal

- In-life: body weights, feed consumption, clinical observations
- Necropsy on pnd 18: body weight, liver weight, thyroid weight, uterine implantation sites counted (for postimplantation prenatal loss), blood samples for T4/TSH; thyroid retained in fixative for possible subsequent histopathology

Offspring

- Apparent sex ratio (by measured anogenital distance on pnd 0) with body weight on pnd 0 (at birth), and on pnd 4, 7, 14, 18, and 21, 22, 42, or 52 (anogenital distance not measured)
- Postnatal survival and development
- Cull litters to 6 females and 4 males on pnd 4.
- At necropsy on pnd 4: gross reproductive tract anomalies (e.g., hypospadias), missing, small, or ectopic testes or ovaries, missing or small epididymides, missing seminal vesicles or oviducts, presence of Wolffian ducts or their derivatives (epididymides and seminal vesicles) in females, presence of Müllerian ducts or their derivatives (oviducts) in males, weigh uterus
- On pnd 12-13, examine males for retained nipples
- On pnd 18, three females per litter are chosen and dosed sc with the test compound until pnd 21. Females are necropsied 6 hours past the last dose, and body, ovary, and uterine wet weights are determined. The reproductive system is examined for anomalies. Serum is assayed for E2, and uterine histology is done on 1 female per litter.
- On pnd 18, 2 males per litter are selected. Necropsy may occur on pnd 18 or 22. The reproductive tract is examined for anomalies; weigh body, testes and epididymides, take blood samples for T4/TSH and do thyroid histology.

- Three females per litter are retained postwean and dosed orally from pnd 18 to 42; weight weekly, clinical observations daily, evaluate for acquisition of vaginal patency (VP) (and vaginal threads) starting on pnd 22; do a vaginal smear on pnd 42 and necropsy. Obtain organ weights and do gross pathology
- Two males per litter are retained postwean and dosed orally from pnd 18 to 52; weight weekly, clinical observations daily, evaluate for acquisition of preputial separation (PPS) starting on pnd 35; necropsy on pnd 52. Obtain organ weights and do gross pathology, serum T4 and TSH, histology on the testes, epididymides, and thyroid.

4.3.3.9 Relevance of Data Collected and Associated Endpoints. As discussed previously, all of the endpoints identified in the Kavlock et al. *in utero*/lactational screening assay are relevant to the identification of endocrine disrupting compounds.

4.3.3.10 Known False Negatives, Known False Positives. This protocol has not been tested.

4.3.3.11 Sensitivity of Assay, Lowest Level of Detection. This protocol has not been tested. However, serum estradiol, thyroxine, and thyroid-stimulating hormone (TSH) may provide a sensitive measure of perturbation of the endocrine system.

4.3.3.12 Specificity of Assay. Likely to be non-specific.

4.3.3.13 Statistical or Non-statistical Methods Used for Analysis. The proposed statistical methods have not been identified. However, it is desirable to use a treatment group size large enough to perform parametric statistics. Dose-related trends, significant dose effects, and comparison to the vehicle control group are appropriate (see Section 6.8).

4.3.3.14 Decision Criteria Used to Classify a Test Chemical. An endocrine sensitive parameter with a statistically significant ($p<0.05$) dose effect and a significant pairwise comparison between one dose level and the control group, in the presence of a significant dose-related trend, would be the strongest indication of test chemical effect at that dose level. However, observational data must also be taken into account in order to determine whether the numerical data is a reasonable reflection of the biological results.

4.3.4 Strengths of the Protocol C Study Design

4.3.4.1 Overall Protocol Design. The protocol provides for enough animals to evaluate each endpoint. The endpoints are logical, appropriate, and can be evaluated within the protocol design. However, the protocol does not minimize the use of animals.

4.3.4.2 Route of Administration/exposure. The proposed route of administration of the test compound is oral. The oral route of administration is appropriate, mimicking a likely route of exposure to endocrine-disrupting compounds that is anticipated to occur in humans. Dosed feed or drinking water would provide a relevant exposure model. However, exposure using dosed feed or drinking water is variable, and depending on the animal's aversion to the chemical, may be inaccurate. In addition, the use of dosed feed or water provides an opportunity for pre-

weanling animals to be directly exposed as they begin to self-feed, and are weaned, in addition to the compound that is transferred through lactation of the mother. Thus, gavage, although not the best model of human exposure, is the optimal choice for oral exposure in this protocol.

4.3.4.3 Dosing Period/duration. Exposure is to be to the pregnant dams on gd 6 through pnd 18 (weaning). The period of administration is appropriate in that it encompasses the critical *in utero* and lactational periods defined by other studies that have identified endocrine-disrupting compounds.

4.3.4.4 Measurements/endpoints. This assay includes the following endpoints/measurements: AGD; uterotrophic activity in immature females; E2; uterine histology; T4; TSH; thyroid histology; organ weights; vaginal cytology; vaginal patency; PPS; retained nipples; and male reproductive histology. These endpoints have been shown to be appropriate and sensitive to endocrine-disrupting activities in the rodent in previous studies.

4.3.5 Weaknesses of the Protocol C Assay

4.3.5.1 Overall Protocol Design. The protocol seems quite complex, as it tries to be all things for all people. By distributing pups within litters to the various cohorts, the protocol was designed to look at endpoints examined in the original Tier 1 screening assays: male and female pubertal effects, uterotrophic effects, and thyroid effects. However, in this protocol, the F1 offspring are exposed at least potentially by transplacental and translactational processes, since the F0 maternal animal is dosed from gd 6 through wean on pnd 18.

4.3.5.2 Route of Administration/exposure. No weaknesses found.

4.3.5.3 Dosing Period/duration. No weaknesses found.

4.3.5.4 Measurements/endpoints. The endpoints evaluated in this assay need to more evenly address both male and female effects. For instance, thyroid hormones are scheduled to be evaluated only in males. In addition, ovarian histology is not included in the protocol. Female thyroid hormone evaluation and histology, and ovarian histology should be included. In addition, it is questionable whether the uterotrophic assay is a useful addition to this screening protocol, or whether the female selected for this endpoint could be used to determine more directly affected changes.

4.3.6 Test Method Performance - Statistical Power of Approach

The statistical power of the approach is adequate.

4.3.7 Test Method Reliability - Assurance of *in Vivo* Biological Test Vigor, Sensitivity, and Reproducibility

The endpoints proposed in this protocol have been shown to be sensitive and reproducible. It is likely that use of this protocol will provide a database of information that will be comparable across studies and across different compounds.

4.4 RELATED STUDY DESIGNS

4.4.1 Description of Lactational Exposure Studies

Lactational exposure studies are those in which the pup is potentially exposed to the test compound during the period of lactation (pnd 0-21 for rats; pnd 0-18 for mice). This is usually interpreted as being exposure to the compound through the milk, the maternal animal having been directly exposed to the compound. This model is most appropriate for compounds that may be sequestered in the mother's body and mobilized from fat stores during lactation, or those that the mother ingests intentionally, such as medicines or nutritional supplements. Compounds that reach the pup lactationally have likely been metabolized by the mother prior to being excreted in the milk.

4.4.2 Description of Multigenerational Exposure Studies

The 2-generation, such as that required for registration of a chemical with the EPA (TSCA 799.9380, August 15, 1997; OPPTS 870.3800, Public Draft, February, 1996; OECD No. 416, 1983; FIFRA, Subdivision F, Guidelines 83-4) is designed to evaluate the effects of a chemical on reproduction and fertility through two generations, with one litter per generation. If necessary, a third generation may be produced. Gonadal function, estrous cycles, mating behavior, fertilization, implantation, pregnancy, parturition, lactation, weaning, and the offsprings' ability to achieve adulthood and successfully reproduce through two generations are all endpoints for this study. In addition, neonatal survival, growth, and development are also evaluated. Hormonally-induced effects such as abortion, resorption, or premature delivery, in addition to physically observable abnormalities, such as masculinization of the female offspring or feminization of the male offspring, can be detected. This study design is most applicable to situations where chronic exposure to a compound is likely to occur over several generations. Dosing begins with a 10-week pre-breeding exposure (weanling animals) and continues until necropsy of the final generation.

4.4.3 Description of Studies Using Direct Exposure to the Pups

Direct exposure of the pups in a study can be accomplished by gavaging the animals beginning on pnd 7 and ending at weaning (pnd 21 for rats, pnd 18 for mice). This provides a known exposure to the postnatal animal, and has the advantage of not being dependent upon lactational transfer. However, because of the undeveloped nature of the pups metabolic systems, and the fact that by direct dosing, the pup receives the compound without benefit of metabolism by the maternal organism, a different spectrum of toxicity may be observed. This model is most useful for test compounds that would be given directly to human babies and children, such as medicines, nutritional supplements, or environmental contaminants as they may absorbed through drinking water or food.

4.4.4 Discussion of Related Study Designs

4.4.4.1 Species Tested, Including Specifics on Strain or Stock, Sex, Age Range, Diet, Etc.

These study designs have been conducted most frequently with rats and mice. The most common strain of rat is the Sprague-Dawley rat. Long-Evans hooded rats are also used. The

CD-1 mouse is the most commonly used strain of mouse. Standard laboratory chow is used. Age at initiation of dosing is specific to the study design (see above).

4.4.4.2 Chemical/product Evaluated, Including Chemical/product Name (If Mixture, Provide Information on All Components), Cas Registry Number, Chemical/product Class, Physical/chemical Characteristics Directly Related to Test Performance (E.g., Water and Lipid Solubility, Ph, Pka, Etc.), Stability of Test Material in Test Medium, Purity, Supplier/source. A wide variety of compounds have been evaluated using these study designs.

4.4.4.3 Route of Administration. Gavage is the most desired route of administration for the lactational and direct pup exposure study design. This provides a standardized dose of the compound either to the mother or the pup, with no extraneous exposure from food, water, etc. Dosed feed and dosed drinking water are the most commonly used routes of administration for the 2- or multi-generation studies. Since exposure continues uninterrupted from the beginning of the study until the last necropsy, the pup access to dosed food or water is not an adverse experimental factor.

4.4.4.4 Doses Used (Including Vehicle Controls). These study designs are most often conducted with a vehicle control and three dose levels.

4.4.4.5 Duration of Exposure. See above.

4.4.4.6 Appropriate Vehicle, Negative And/or Positive Controls; Basis for Selection. An appropriate vehicle would be one that has no endocrine activity or other overt toxicity, and that provides a stable dose formulation that can be accurately analyzed for test compound concentration.

4.4.4.7 Endpoints Measured. The endpoints listed below are those required by current EPA guidelines for the 2-generation study design. For the other study designs, only those parameters having to do with maternal status and pup reproductive development until puberty are applicable.

- Gonad development (size, morphology, weight) > accessory sex organ (ASO) development
- ASO weight ± fluid; histology
- Sexual development and maturation: acquisition of vaginal patency (VP), pre-putial separation (PPS)
- Fertility
- Fecundity
- Time to mating
- Estrous cyclicity
- Gestation length
- Abortion
- Premature delivery
- Dystocia
- Spermatogenesis
- Epididymal sperm numbers, motility, and morphology; testicular homogenization-resistant spermatid head counts; daily sperm production (DSP); efficiency of DSP

- Gross and histopathology of reproductive tissues
- Anomalies of the genital tract
- Viability of the conceptus *in utero* (prenatal demise)
- Survival and growth of offspring
- Anogenital distance (AGD), currently measured in F2 pups at birth if "triggered" by effects observed in F1 offspring development
- Maternal lactational behaviors (e.g., nursing, pup retrieval, etc.)
- Altered apparent sex ratio (based on AGD)
- Malformations of the urogenital system
- Altered sexual behavior
- Changes in testis and accessory sex organ weights
- Retained nipples in male offspring
- Altered AGD (now triggered from PPS/VP)
- Male fertility
- Agenesis of prostate
- Changes in androgen-dependent tissues in pups and adults (not limited to sex accessory glands)
- Grown, body weight
- Food consumption, food efficiency
- Developmental abnormalities

4.4.4.8 Data Collected. See above.

4.4.4.9 Relevance of Data Collected and Associated Endpoints. Interpretation of data most sensitive to developmental endocrine disruption:

- If anogenital distance changes (co-vary by body weight for statistical analysis):
  - increased in females from androgen
  - decreased in males from anti-androgen
- Retained areolas in males: from anti-androgen
- If uterine weight changes:
  - increased precociously by estrogen
- early vaginal opening: increased precociously by estrogen
- If there are male reproductive tract anomalies: from anti-androgens
- If there are female reproductive anomalies (or male accessory tissues): from androgens, estrogens, or possibly anti-estrogens
- If changes in T4/TSH, thyroid weight (histopathology): from thyroid or anti-thyroid activity
- If change in age at acquisition of VP/PPS: accelerated VP from estrogens, delayed VP from anti-estrogens/androgens, accelerated PPS from androgen, delayed PPS from anti-androgen (co-vary age at VP or PPS by body weight at acquisition for statistical analysis)

4.4.4.10 Known False Negatives, Known False Positives. There is little chance of producing a false positive with the 2- or multi-generation study design, since many aspects of

reproductive development and function are evaluated. A false negative could result if exposure were limited to lactation only, or by direct administration to the pups during the lactational period, if the endocrine disrupting activity of the test compound were dependent upon prenatal targets.

4.4.4.11 Sensitivity of Assay, Lowest Level of Detection. Ranges from very sensitive to insensitive, depending on the parameter.

4.4.4.12 Statistical or Non-statistical Methods Used for Analysis. Parametric statistics are most applicable.

4.4.4.13 Decision Criteria Used to Classify a Test Chemical. Statistically significant ($p<0.05$) effect of test compound on one or more of the endocrine-sensitive endpoints, in conjunction with other observations in the study.

5.0 RECOMMENDED STUDY DESIGN

The recommended study design is Protocol B. This protocol combines the best of the other two protocols in that it uses the dosing duration of the Segment III study (Study A; gd 6 to pnd 21) and it evaluates the postnatal consequences of in utero exposure to the acquisition of male and female puberty and thyroid-specific endpoints, without employing the logistical complexity of Protocol C.

5.1 SPECIES

The Sprague-Dawley outbred rat and the Swiss outbred mouse are considered as the animals of choice for the proposed study design for the following reasons:

1. The Sprague Dawley outbred rat has been chosen over another strain frequently used in toxicity studies, the Fischer 344 (F344) inbred albino rat. The F344 rat was specifically excluded from use in the early version of the U.S. EPA Health Effects Test Guidelines, OPPTS 870.3800, Reproduction and Fertility Effects (U.S. EPA, 1994 Draft), due to its low fecundity. In addition, this strain shows early onset and increased incidence of spontaneous male reproductive tract lesions, including testicular cancers, relative to the Sprague-Dawley, Wistar, or Long-Evans outbred strains. In subsequent versions of the OPPTS Health Effects Test Guidelines, Reproductive and Fertility Effects (e.g., the 1996 "Public Draft" and the final OPPTS Health Effects Test Guidelines in 1998), the specification of F344 for exclusion was deleted, but the strong suggestion was retained that rat strains with poor fertility, fecundity, and high spontaneous incidence of reproductive lesions should not be used. However, the F344 rat is specifically excluded from the U.S. EPA Health Effects Test Guidelines for developmental neurotoxicity (OPPTS 870.6300). In contrast, the Sprague-Dawley rat is commonly used for reproductive and developmental toxicity evaluations, as witnessed by published historical data from 15 participating laboratories in the research contract and industrial sectors (Charles River, 1993,

1996). Such a large historical database of reproductive and developmental toxicity studies does not exist for the Wistar or Long-Evans strains.

2. The F344 rat pup is small in size relative to the outbred strains. On pnd 21, the average weight of CDF® (F-344) male pups is 25-35 g as compared to 40-60 g for Sprague-Dawley rats of the same age [Crl:CD®(SD) rats; Charles River, 1999]. Although Charles River currently recommends weaning the F344 rat pups on pnd 21 (Charles River, 1999), this strain has historically been weaned on pnd 28 to ensure that the pups are large enough to successfully access feed and drinking water supplied through the cage lid of plastic "shoebox" cages. The weight of the F344 rat pup on pnd 28 is equivalent to that of a Sprague-Dawley rat pup on pnd 21. Therefore, it may not be feasible to wean F344 rat pups on pnd 21, as is specified in the protocol.

3. Small litter size (6 pups per litter; Charles River, 1999) is another compelling reason to avoid the F344 rat for the proposed study design. The Sprague Dawley rats has an average litter size of 11 pups (Charles River, 1999). Thus, reproductive and developmental characteristics (especially litter size and postnatal growth patterns) for the Sprague-Dawley rat appear more suitable for the proposed study design.

With regard to the Swiss albino mouse, a large data base of reproductive and developmental indices is available (Charles River, 1996). Similar to the Sprague-Dawley rat, the Swiss mouse has the reproductive and growth characteristics of high fertility and large litter size that are appropriate for use in the proposed study design.

5.2 TEST CHEMICALS APPLICABLE TO ASSAY

Any suspected endocrine disruptor that does not cause overt maternal toxicity, interfere with nutritional status, and does not severely affect post-implantation or postnatal viability of the embryos would be applicable. Compounds that cross the placenta and are secreted in the breast milk would be best served by this assay.

5.3 ROUTE AND DURATION OF EXPOSURE

Oral administration by gavage; administer to the mother from implantation (gd 6) through weaning (pnd 21). In a screening study, parenteral routes of administration may be considered, since the study is designed to determine the intrinsic activity of the compound. With regard to ip administration in pregnant animals, the following caveats apply:

- There is risk of inadvertent injection into the gravid uterus, especially late in pregnancy, with unanticipated, inadvertent, and untoward direct exposure to the conceptus(es).
- Irritation of the uterine wall may occur, leading to adverse consequences during pregnancy, which further compounds data interpretation.
- The test material in the abdominal cavity may be absorbed through the uterine wall, with subsequent direct exposure to the conceptus(es).

- In a neonatal rat or mouse, the abdominal cavity is so small and the abdominal organs so relatively large, that there is a risk of inadvertent injection directly into the gastrointestinal tract, kidneys, urinary bladder, or abdominal testes (prior to testes descent on pnd 15-20).
- If the dam detects the injection site on the pup as she grooms it (especially if there is blood at the site), she will usually kill and ingest the pup.
- Finally, ip injection is clearly not a route of administration relevant to human or environmental wildlife exposure.

If such a direct parenteral route is needed in a mechanistic study, iv, sc, or im injection is much better. To simulate human or wildlife exposure, routes such as dosed feed or dosed drinking water, are considered appropriate and relevant routes of administration. Dosed feed or drinking water would provide a relevant exposure model. However, exposure using dosed feed or drinking water is variable, and depending on the animal's aversion to the chemical, may be inaccurate. In addition, the use of dosed feed or water provides an opportunity for pre-weanling animals to be directly exposed as they begin to self-feed and are weaned, in addition to the compound that is transferred through lactation of the mother. Thus, gavage, although not the best model of human exposure, is the optimal choice for oral exposure in this protocol. If the proposed model of human exposure is by ingestion through food and/or drink, gavage administration to animals may indeed optimally approximate the eating patterns of people.

5.4 TEST CONDITIONS

Standard laboratory test conditions suitable for government guideline tests.

5.5 ENDPOINTS

Maternal

- In-life: body weights, feed consumption, clinical observations
- Necropsy: body weight, liver weight, thyroid weight, postimplantation prenatal loss, blood T4/TSH; possible thyroid histopathology

Offspring

- Apparent sex ratio on pnd 0; with body weight daily from birth until weaning
- Postnatal survival and development
- Reproductive tract anomalies (including inappropriate retention of embryonic anlagen and malformations of gonads, etc.) and uterine weight on pnd 4
- Nipple retention in preweanling males
- Female reproductive tract anomalies and uterine weight, possible uterine histopathology on pnd 14 (this postnatal day of necropsy will detect precocious uterine enlargement, i.e., a uterotrophic response, prior to the beginning of the naturally-occurring process.
- Male and female reproductive tract anomalies, weight of testis, epididymides, uterus, and ovaries; examine for precocious puberty (acquisition of vaginal patency) and vaginal threads, T4/TSH
- Postwean weight weekly, clinical observations daily, acquisition of VP (and vaginal threads) for females and PPS for males

- On day of acquisition of VP and PPS, weigh animals and necropsy, examine as on pnd 21, weigh and retain thyroid, testes, epididymides, ovaries, and uterus; T4/TSH, E2, and T. Note: although E2 and T are both released in a cyclical pattern, we are recommending a single sampling at necropsy at the time of acquisition of puberty. To properly characterize the normal (or abnormal) fluctuations of these hormones in blood would be best done by cannulating animals and taking serial samples over time - this is expensive, time consuming, and technically difficult, and therefore was not included in this screening assay. A single sampling of E2 and T levels which should be at their peaks at acquisition of puberty, in the context of the other endpoint collected, should be sufficient to identify compounds interacting with the endocrine system by any of a large number of mechanisms.

5.6 DATA COLLECTED

Maternal

- In-life: body weights, feed consumption, clinical observations
- Necropsy: body weight, liver weight, thyroid weight, uterine implantation sites counted (for postimplantation prenatal loss), blood samples for T4/TSH; thyroid retained in fixative for possible thyroid histopathology

Offspring

- Apparent sex ratio (by measured anogenital distance on pnd 0) with body weight on pnd 0 (at birth), and on pnd 4, 7, 14, and 21 (anogenital distance not measured)
- Postnatal survival and development
- At necropsy on pnd 0 and 4: reproductive tract anomalies (e.g., hypospadias), missing, small, or ectopic testes or ovaries, missing or small epididymides, missing seminal vesicles or oviducts, presence of Wolffian ducts or their derivatives (epididymides and seminal vesicles) in females, presence of Müllerian ducts or their derivatives (oviducts) in males, weigh uterus
- On pnd 10-12, examine males for retained nipples
- On pnd 14, necropsy females (one/litter), weigh uterus (possible histopathology to measure uterine gland number and luminal epithelial cell height), examine reproductive system for anomalies
- On pnd 21, necropsy one/sex/litter or all remaining pups, examine males for reproductive tract anomalies, weigh testes and epididymides, examine females for reproductive tract anomalies, weigh uterus and ovaries, examine for precocious puberty (acquisition of vaginal patency) and vaginal threads, take blood samples for T4/TSH
- If pups are retained postwean, weight weekly, clinical observations daily, evaluate for acquisition of VP (and vaginal threads) for females starting on pnd 22, evaluate for acquisition of PPS for males starting on pnd 30
- On day of acquisition of VP and PPS, weigh animals and necropsy, examine as on pnd 21, also weigh and retain thyroid, testes, epididymides, ovaries, and uterus; take blood samples for T4/TSH, E2, and T

5.7 RELEVANCE OF DATA COLLECTED AND ASSOCIATED ENDPOINTS

Interpretation of Endpoint Changes

- If anogenital distance changes (covary by body weight for statistical analysis):
  - increased in females from androgen
  - decreased in males from estrogen or anti-androgen
- If uterine weight changes:
  - increased precociously by estrogen
- If there are male reproductive tract anomalies: from anti-androgens or possibly estrogens (feminization)
- If there are female reproductive anomalies: from androgens, estrogens, or possibly anti-estrogens
- If changes in T4/TSH, thyroid weight (histopathology): from thyroid or anti-thyroid activity
- If there is an increase in the number of nipples in males: antiandrogenic activity
- If change in age at acquisition of VP/PPS: accelerated VP from estrogens, delayed VP from anti-estrogens/androgens, accelerated PPS from androgen, delayed PPS from anti-androgen (covary age at VP or PPS by body weight at acquisition for statistical analysis)

5.8 DATA ANALYSIS

The proposed statistical methods have not been identified. However, it is desirable to use a treatment group large enough to perform parametric statistics. Dose-related trends, significant dose effects, and comparison to the vehicle control group are appropriate. A discussion of statistical methods follows. Specific computer software has been identified. However, any software that is capable of accomplishing the proposed analyses would be appropriate.

Independent Continuous Data. This type of data is measured on animals that are statistically independent (i.e., not from the same litters) and includes such continuous endpoints as body weights, feed consumption, organ weights, time to mating, and length of gestation. Treatment groups will be compared to the concurrent control group using either parametric ANOVA under the standard assumptions or robust regression methods (Zeger and Liang, 1986; Royall, 1986; Huber, 1967) which do not assume homogeneity of variance or normality. The homogeneity of variance assumption will be examined via Levene's test (Levene, 1960). If Levene's Test indicates lack of homogeneity of variance ($p<0.05$), robust regression methods will be used to test all treatment effects. The robust regression methods use variance estimators that make no assumptions regarding homogeneity of variance or normality of the data. They will be used to test for linear trends across dose as well as overall treatment group differences (via Wald chi-square tests). Significant overall treatment effects will be followed by single degree-of-freedom t-tests for exposed vs. control group comparisons, if the overall treatment effect is significant. If Levene's test does not reject the hypothesis of homogeneous variances, standard ANOVA techniques will be applied for comparing the treatment groups. The GLM procedure in SAS® 8 (SAS Institute Inc., 1999a,b,c,d,e, 2000) will be used to test for linear trend, evaluate the overall effect of treatment and, when a significant treatment effect is present, to compare each exposed group to control via Dunnett's Test (Dunnett, 1955, 1964). Standard ANOVA methods, as well as Levene's Test, are available in the GLM procedure of SAS®

Version 8, and the robust regression methods are available in the REGRESS procedure of SUDAAN® Release 8.0 (RTI, 2001). Both packages are currently in use on GLP studies.

Independent Binary Data. This type of data is measured on animals that are statistically independent (i.e., not from the same litters) and includes binary endpoints such as the parental reproductive indices (e.g., mating, fertility, and live litter indices). All indices will be analyzed by Fisher's Exact test for overall heterogeneity among treatment groups and by an exact version of the Cochran-Armitage test for linear trend on proportions (Cochran, 1954; Armitage, 1955; Agresti, 1990). When the overall Fisher's Exact test is significant ($p<0.05$), pairwise comparisons of individual exposed groups vs. control are performed using pairwise Fisher's Exact tests. All of these tests can be obtained via the FREQ procedure in SAS 8. The SAS MULTTEST procedure can be used to obtain p-value adjustments for the multiple treatment comparisons resulting from repeated applications of Fisher's Exact test. The p-value adjustments are based on the bootstrap and permutation resampling techniques of Westfall and Young (1993).

Cluster-Correlated Data. Cluster-correlated data are those which are measured on more than one pup/sex/litter. Such endpoints include, for example, body and organ weights of weanlings at necropsy, skeletal and visceral malformations of pups, lactational pup body weights, periodic pup survival indices, and the anogenital distance (a developmental landmark). GEE regression methods (Zeger and Liang, 1986; Liang and Zeger, 1986) in SAS® 8 or SUDAAN® 8.0 will be used to evaluate overall significance, test for linear trend across dose groups, and test pairwise comparisons to the control group values.

Some of these outcomes are continuous (e.g., body and organ weights, anogenital distance) and some are binary (e.g., pup malformations, periodic pup survival). Ordinal outcomes would include those measured on a severity scale, such as none, mild, moderate, and severe.

No matter what type of endpoint is examined (e.g., continuous, binary, ordinal), when multiple pups or fetuses from the same litter are used in the analysis, care must be taken to adjust for the resulting correlation of responses within litters. Intracluster correlation, or the tendency for littermates to respond similarly, tends to inflate the true variance of parameter estimates, including percentages and regression coefficients used in these analyses. In other words, fetuses within litters are not statistically independent, and failure to account for this in the statistical analysis will result in underestimated standard errors and false-positive tests for treatment effects (Haseman and Kupper, 1979). Hence, the chance of finding false-positive results is increased when the clustering is ignored.

To incorporate the effects of intracluster correlation and reduce the chances of finding spurious results, a model-fitting method designed specifically for clustered data in which the outcomes may or may not be normally distributed is recommended. Recent advances in analyzing longitudinal and cluster-correlated data for generalized linear models (Zeger and Liang, 1986; Zeger et al., 1988; Lipsitz et al., 1994) have led to new methods for handling binary, categorical, and continuous outcomes on fetuses clustered within litters. The techniques (otherwise referred to as Generalized Estimating Equations, or GEEs) make no strict distributional assumptions about the endpoint of interest (e.g., normality) or the correlations among clustered observations, thereby providing flexibility for a variety of analytical settings.

The generalized linear model provides a unified approach for modeling continuous and non-continuous response variables. For continuous outcomes, the distribution of the responses within litters is assumed to be normal. The expected value of the response is related directly to a linear function of the covariates. For binary outcomes, the distribution of the responses within litters is assumed to be binomial. A logit transformation is used to relate the expected value of the response prob`(Y=1`|`bold x)to a linear function of the covariates (Morgan, 1992). For ordinal outcomes, a multinomial distributional assumption and a cumulative logit transformation is used, leading to the proportional odds model (McCullagh, 1980). The GEE approach requires only specification of the relationship between the mean and variance of the correlated outcomes within each litter. Only the mean, prob`(Y=1`|`bold x) or E```(Y`` line `bold x`), needs to be correctly specified for the estimated regression parameters to be approximately unbiased. Zeger and Liang (1986) and Liang and Zeger (1986) showed how to use GEEs to solve for the regression parameters and their estimated variances in the generalized linear model when the data are cluster-correlated. In the linear and logistic regression setting, the model parameters can be estimated in the usual way, using standard methods such as maximum likelihood or ordinary least squares. These estimates are identical to those obtained if the data were independent, and they are known to have desirable statistical properties even under cluster sampling (e.g., they are asymptotically normal and unbiased). Robust variance estimates of model parameters fully account for the intracluster correlation of responses. This technique is valid for any underlying correlation structure within a litter, as long as the litters are statistically independent. In order to gain efficiency (i.e., reduce the variance of estimated parameters), a pairwise correlation model for the fetuses within a litter can be specified, and estimates of the correlation parameters are used in the estimation of regression coefficients. However, the correlation pattern is treated as a nuisance parameter, and not explicitly included in the regression model. The regression coefficient estimates and their robust variance estimates are approximately unbiased regardless of whether or not the correlation structure is correctly specified.

Many correlation structures have been developed for this purpose, with working independence implying no correlation within litters, an exchangeable structure denoting equal pairwise correlations for all pairs of fetuses within a litter (popular for developmental toxicology studies), and a time-dependent structure (e.g., auto-regressive or m-dependent) used frequently for longitudinal designs. When the working correlation structure is consistent with empirical correlations, efficiency is maximized. However, a robust variance estimator for regression parameters provides valid and unbiased inferences even when the working correlation structure has been mis-specified.

Wald chi-square test statistics are used to evaluate the significance of model parameters relating to treatment effects. For a single degree-of-freedom hypothesis (e.g., high dose vs. control, linear trend), the Wald chi-square test statistic reduces to a standard normal deviate, or the regression coefficient estimate divided by its standard error. For multiple degree-of-freedom hypotheses (e.g., the overall effect of treatment), the Wald chi-square test is analogous to the ANOVA F-test for linear models with continuous responses.

The application of the estimating equation approach to developmental toxicology studies has been demonstrated by many authors, among them Bieler and Williams (1995), Liang and

Hanfelt (1994), Carr and Portier (1993), Lefkopoulou et al. (1989), Lockhart et al. (1992), Rao and Colin (1991), and Williams (1982).

Software for implementing the GEE approach can be found in the GENMOD procedure of SAS® 8 (SAS Institute Inc., 1999a,b,c,d,e, 2000) as well as SUDAAN® 8.0 (RTI, 2001). SUDAAN was developed at RTI, and fits linear, logistic, multinomial logistic, log-linear, and proportional hazards models to clustered and longitudinal data. Both independent and exchangeable working correlation structures are available in the REGRESS, LOGISTIC, LOGLINK, and MULTILOG procedures, which fit linear, logistic, log-linear (Poisson models) and multinomial logistic models to continuous, binary, count, ordinal, and nominal categorical data using the methods of Liang and Zeger (1986) and Lipsitz, et al (1994). SUDAAN allows for fixed-effect covariates that are either measured at the fetus (e.g., fetal body weight) or litter levels (e.g., dose group). Cluster sizes can vary, and missing data are assumed to be missing completely at random (meaning that the probability of a missing value is equal for all fetuses within a litter).

6.0 IMPLEMENTATION CONSIDERATIONS

6.1 ESTABLISHMENT OF ASSAY

6.1.1 Ease of Setup and Execution

The selected *In Utero*/Lactational Exposure Assay should be relatively straightforward to set up and execute. Assays such as this have been used at the Environmental Protection Agency, the National Institute of Environmental Health Sciences, and other reputable laboratories for xenoestrogens, environmental antiandrogens, AH receptor agonists, phthalates, and antithyroidal toxicants.

6.1.2 Information on Facilities and Major Fixed Equipment Needed to Conduct the Test

Any facility currently conducting government guideline tests would have the necessary equipment to conduct this test.

6.1.3 General Availability of Other Necessary Equipment and Supplies

All necessary equipment is generally available.

6.1.4 Implementation Considerations

Any laboratory staff currently conducting government guideline tests would have the appropriate training. Additional training may be needed to determine AGD at pnd 0.

6.2 COST/TIME REQUIRED

The duration of the assay is quite long, up to 10 weeks. The cost would be approximately $100,000.

6.3 ANIMAL WELFARE CONSIDERATIONS

6.3.1 Advantages/disadvantages of the Endocrine Disruptor Assays Related to Animal Use

The advantage of using the *In Utero*/Lactational Exposure Endocrine Disruptor Assay with regard to animal use is that the use of a screen, with small numbers of animals, to identify compounds that need further testing would eliminate the wasting of animals on full-blown tests (larger groups sizes) for compounds that may not have a significant level of endocrine-disrupting activity. By using this screen in conjunction with the other Tier I screens, there is a much better chance of identifying compounds that need to be tested, and reducing the overall number of animals that must be used to identify these compounds. By incorporating several viable endpoints to detect disruption of male and/or female reproductive systems, the study design ensures that each animal is used to its fullest extent to provide the most information possible. The proposed protocol would also aid in the selection of endpoints and appropriate F1 animals usage per litter for the Tier 2 multigeneration test. There is no disadvantage related to animal use with respect to this assay, other than the use of animals itself. The selected study design should maximize the data collected from the minimum number of animals necessary.

6.3.2 Rationale for the Need to Use Animals

To date, there is no validated test to replace the use of the whole animal model in the study of mammalian reproduction. Although there are several non-whole animal assays that are critical in identifying mechanism and site of action of an endocrine-disrupting chemical, exposure the whole animal is necessary to evaluate the effect of a suspected endocrine-disrupting chemical on the intricately related processes that define sexual development and maturation.

6.3.3 Type of Animals Required and Rationale

The rat is the species of choice. Previously conducted studies indicate that the rat may be more sensitive than the mouse to the endocrine-disrupting effects of some compounds. Since the Sprague-Dawley rat is the most frequent choice for government guideline studies, and since there are a number of large historical databases describing developmental data with this strain of rat, it would seem that the Sprague-Dawley rat is the best choice for this assay.

6.3.4 Number of Animals Required and Rationale

The number of animals required for this assay depends on several factors: the number of optional endpoints to be evaluated, the type of statistical analysis required, and the cost ceiling for the assay. It is anticipated that a minimum of 10 litters and up to 20 litters per dose group would be required. The study should be conducted with three to five treated groups plus a vehicle control group. Therefore, the number of animals required for this assay would be 40-120 maternal animals. The statistical approach would depend on the results of the Levene's test for homogeneous variances (Levene, 1960) for a given parameter. If Levene's test is not significant (i.e., the variances are homogeneous), then the usual parametric statistics, such as ANOVA, ANCOVA (available on SAS® software; SAS Institute, Inc., 1999a,b,c,d,e, 2000) are appropriate. If Levene's test is significant (i.e., the variances are not homogeneous), then use of

robust regression methods (available on SUDAAN® software; RTI, 2000) is strongly recommended. The larger the group size, the more likely the variances will be homogeneous. Obviously, the use of more animals would increase the cost and effort required for this screening assay. However, this assay has been proposed to either replace a significant number of other Tier 1 screening assays, or to be used as a Tier 1.5 test. Used in this way, the protocol would provide important information to identify low incidence phenomena like malformations and to dose-response assessments over an appropriate range. The power and importance of this protocol are that it is examining adverse effects after exposure during critical developmental windows, but it is much shorter, and therefore less costly than a multigeneration study.

6.3.5 Relative Pain or Distress for Animals

It is not anticipated that the animals would suffer prolonged pain or distress. The proposed route of administration is gavage. The animals may experience some momentary discomfort during the gentle insertion of the gavage needle into the esophagus. However, this lasts only a few seconds and does not result in a lasting effect on the animal. Because the goal of the assay is to evaluate reproductive development and maturation in the offspring of treated mothers, it is anticipated that the doses will be chosen such that there is little overt maternal toxicity and mortality. Should the animals become moribund, they will be sacrificed humanely.

6.4 ASSURANCE OF BIOLOGICAL TEST VIGOR, SENSITIVITY, AND REPRODUCIBILITY

The basic study design of this assay has been conducted previously in other laboratories. It is anticipated that the assay will be sensitive and reproducible.

7.0 DEVELOPMENTAL STATUS OF THE ASSAY

7.1 CURRENT STATUS

The various endpoints included in the proposed assay have been evaluated in other studies. However, the protocol itself has not been validated. Pending a final decision on the study design, the protocol would be ready to enter the pre-validation phase.

7.2 UNCERTAINTIES AND DATA NEEDS

It is desirable to determine whether and how many of the other Tier I assays could be replaced by this assay, and what the relative cost would be. Candidate assays for replacement include the Hershberger assay, uterotrophic assay, pubertal steroidogenesis assay, and/or aromatase assay.

7.3 RECOMMENDATION FOR FURTHER RESEARCH AND DEVELOPMENT

7.4 RECOMMENDATION FOR PRE-VALIDATION STUDIES

Pre-validation studies following the ICCVAM validation process should be initiated. Pre-validation studies should include evaluation of 1-3 chemicals to establish the database for

the validation studies. It is recommended that the study design be performed using positive control compounds that target different pathways in the endocrine system, and for which *in vivo* data already exists. For instance, vinclozolin acts as an anti-androgen, propylthiouracil has anti-thyroid activity, and nonylphenol has estrogenic activity. Using positive control compounds with differing mechanisms of action will aid in the development of the pre-validation database for the assay.

7.5 RECOMMENDATION FOR VALIDATION STUDIES

Validation of the study design through interlaboratory comparisons should be conducted with compounds that span the possible endocrine effects, including strong and weak androgen receptor agonists and antagonists, estrogen receptor agonists, steroidogenesis inhibitors, and aromatase inhibitors.

Table 4. Selected Studies of GnRH Modulators

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
perphenazine	1, 10, or 25 mg/kg; 2x/d; 14, 17, 20, or 36d; sc	Rat Sprague Dawley	no effect on vaginal patency or age of initiation of vaginal estrus or proestrus; delayed onset of first regular estrous cycle which persisted after treatment ended; decr serum 3,5,'-triiodothyronine & thyroxine & incr serum prolactin in 10 & 25 mg/kg grps; incr serum thyroid stimulating hormone levels in females		Greenley et al. 1982
gonadotropic releasing hormone agonist	10 mg/kg; pnd 2, 5; sc	Rat Wistar	decr testis wt at days 18- 75; apical immunolocalization of AQP-1 to efferent duct epithelium, slight decr epithelial cell height on day 18; delayed flattening of rete testis epithelial cells on day 18		Fisher et al. 1999
atrazine	12.5, 25, 50, 100, 150, or 200 mg/kg/day; pnd 23-53; po	Rat Wistar	delayed preputial separation at all doses, reduced ventral prostate weight (50 mg/kg/day and above); pair-fed group mimicked effects at high dose.	Direct CNS effect	Stoker et al., 2000b
testosterone propionate	2.5 mg/animal (immature); 1x; sc	Rat Long Evans	in immature grp incr ventral prostate wt	lack of effect in immature rats indicates they likely haven't started releasing anterior pituitary hormone	Bari et al. 1984
testosterone propionate	12.5 mg/animal (mature); 1x; sc	Rat Long Evans	in mature grp, decr testes wt; incr mean seminal vesicle & ventral prostate wt; incr seminiferous tubules & non sperm tubules	decr testes wt probably due to suppressive effect on release of anterior pituitary gonadotrophins w/ eventual suppression of testosterone secretion by atrophied Leydig cells	Bari et al. 1984
testosterone propionate	0.5 mg/animal (immature); 1x/d; 15d; sc	Rat Long Evans	in immature grp decr testes wt; incr seminal vesicle & ventral prostate wt.; incr seminiferous tubules & non sperm containing tubules; decr sperm containing tubules; gross atrophy w/ marked irregularity in germ cell pattern & population	decr testes wt probably due to suppressive effect on release of anterior pituitary gonadotrophins w/ eventual suppression of testosterone secretion by atrophied Leydig cells	Bari et al. 1984

Table 4. Selected Studies of GnRH Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
testosterone propionate	2.5 mg/animal (mature); 1x/d; 15d; sc	Rat Long Evans	in mature grp, decr testes wt; incr mean seminal vesicle & ventral prostate wt; incr seminiferous tubules & non sperm tubules; decr sperm containing tubules	decr testes wt probably due to suppressive effect on release of anterior pituitary gonadotrophins w/ eventual suppression of testosterone secretion by atrophied Leydig cells	Bari et al. 1984

γ-GT = γ-glutamyl dehydrogenase
AR = androgen receptor
CNS = central nervous system
d = days
decr = decreased
FSH = follicle stimulating hormone
gd = gestational day
grp = grp
GSH = glutathione
hCG = human chorionic gonadotropic
inc = increased
ld = lactation day
LDH = lactate dehydrogenase
LH = luteinizing hormone
pc = post coitum
pd = postpartum day
pnd = postnatal day
po = gavage
PPS = preputial separation
sc = subcutaneous
SDH = sorbitol dehydrogenase
TSH = thyroid stimulating hormone
VO = vaginal opening
w/o = without
w/ = with
wks = weeks
wt = weight

Table 5. Selected Studies of Estrogen Modulators

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
methoxychlor	100, 200 mg/kg/d; pnd 21 to 77-80 (males); po	Rat Long Evans	decr body wt wk 4 (200 mg/kg) & 5-11 and necropsy (100 & 200 mg/kg); in both grps delayed PPS, decr liver, kidney, seminal vesicle wt, caudal epididymal wt, caudal sperm count, incr adrenal wt; in 200 mg/kg grp decr wt at PPS, testis wt, pituitary wt, & testicular lesions in 2 rats		Gray et al. 1988
methoxychlor	100, 200 mg/kg/d; pnd 21 to ppd 1 (females); po	Rat Long Evans	in both grps decr body wt wk 4-10 and necropsy; accelerated VO and 1st estrous smear, decr % vaginal smears w/ leukocytes at VO, incr cornified vaginal smears at 1st estrous, decr corpora lutea; decr % fertility, decr no. live pups pnd 1; in 200 mg/kg grp decr no. live pups pnd 5		Gray et al. 1988
methoxychlor	25, 50 mg/kg/d; pnd 21 to 80-85 (males); pnd 21 to ppd 15 (females); po	Rat Long Evans	in both grps decr body wt at 10 & 11 wks, necropsy body wt; in 50 mg/kg grp decr body wt wk 9, decr seminal vesical & caudal epididymis wt, decr caudal sperm count; in both grps incr pituitary prolactin		Gray et al. 1988
methoxychlor	25, 50 mg/kg/d; pnd 21 to ppd 15 (females); po	Rat Long Evans	in both grps accelerated VO, age at 1st estrous, decr wt at VO; in 25 mg/kg grp decr age at 1st cycle; in 50 mg/kg grp decr % leukocytes in vaginal smears, incr cornified smears at 1st estrus, incr pup wt pnd 1		Gray et al. 1988
methoxychlor	100, 200 mg/kg/d; pnd 21 to 97-100(males); po	Rat Long Evans	in both grps delayed PPS; decr body wt at necropsy, decr liver, kidney, seminal vesicle, testes, caudal epididymis, & pituitary wt; decr caudal sperm count; in 200mg/kg grp incr adrenal wt; in both grps decr sperm TSH; incr pituitary prolactin, FSH, & TSH; in both grps decr seminiferous tubular testosterone, decr initial hCG-stimulated testosterone; in 200 mg/kg grp decr hCG-stimulated testosterone at 3 hr; in 100 mg/kg grp decr interstitial fluid & serum testosterone		Gray et al. 1988

Table 5. Selected Studies of Estrogen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	methoxychlor	100, 200 mg/kg/d; pnd 21 to ppd 15 (females); po	Rat Long Evans	in both grps incr adrenal wt, decr ovarian & pituitary wt; in 200 mg/kg grp decr necropsy body wt, liver wt; in both grps accelerated VO, age at 1st estrus, decr wt at VO, decr % vaginal smears w/ leukocytes; incr no. cornified smears at 1st estrus, decr fertility, decr no. live pups on pnd 1 & 5; in 200mg/kg grp incr age at 1st cycle; in 100 mg/kg grp incr pup wt pnd 1		Gray et al. 1988
2	methoxychlor	50 mg/kg/d; pnd 21 - lactation; (females); po	Rat Long Evans	F1 female offspring accelerated VO, decr no. pups at day 35-253, decr no. litters, decr % normal cyclicity at 11 mo, incr liver wt, incr paired adrenal wt, incr rt kidney wt, incr pituitary tumors		Gray et al. 1988
3	estradiol	10 μg; 2d; sc	Rat Sprague Dawley	incr uterine wt; induced lordosis behavior		Gray et al. 1999a
4	octylphenol	200 mg/kg/d; 2d; ovarianectomized; sc	Rat Sprague Dawley	incr uterine wt; induced lordosis behavior		Gray et al. 1999a
5	methoxychlor	200, 400 mg/kg/d; 2d; ovarianectomized; sc	Rat Sprague Dawley	incr uterine wt; induced lordosis behavior (400 mg/kg)		Gray et al. 1999a
6	methoxychlor	200, 400 mg/kg/d; 2d; ovarianectomized; po	Rat Sprague Dawley	incr uterine wt; induced lordosis behavior (400 mg/kg)		Gray et al. 1999a
7	methoxychlor	25, 50, 100, 200 mg/kg/d; pnd 21-approx. 150 (females); route not specified	Rat strain not specified	accelerated VO & age at first estrus in all grps; altered estrous cyclicity; incr adrenal wt & decr pituitary, paired ovary wt, no. of implantation sites, fertility & litter size in 100 & 200 mg/kg/d grps; inhibited ovarian luteal function, blocked implantation; decr offspring fertility at 50 mg/kg; incr ovarian histology effects in offspring at 100 mg/kg; incr running activity & uterine histology effects in offspring at 200 mg/kg		Gray et al. 1988

Table 5. Selected Studies of Estrogen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
methoxychlor)	25, 50, 100, 200 mg/kg/d; pnd 21-100 (males); route not specified	Rat strain not specified	incr adrenal wt, accelerated PPS, & decr seminal vesicle wt in 100 & 200 mg/kg grp; decr seminal vesicle wt in 50 mg/kg grp; decr wt at PPS, testis wt, serum testosterone, & serum ABP in 100 mg/kg grp; decr hCG stimulated testosterone; decr caudal epididymal wt & sperm count at 50, 100, & 200 mg/kg grp; decr epididymal testosterone, pituitary wt, & serum TSH & incr pituitary FSH & TSH in 100 & 200 mg/kg grp; incr pituitary prolactin all grps		Gray et al. 1988
lindane	10, 20 mg/kg/d; pnd 21-approx. 150 (females); route not specified	Rat strain not specified	decr % normal cyclicity & running wheel activity in 20 mg/kg grp, decr litter size in both dose grps		Gray et al. 1988
lindane	10, 20 mg/kg/d; pnd 21-100 (males); route not specified	Rat strain not specified	no effect on reproductive development		Gray et al. 1988
Aroclor 1221	1 or 10 mg; pnd 2-3; sc	Rat Sprague Dawley	accelerated VO; persistent vaginal estrus at 6 mo, & incr % anovulatory at sac in 10 mg grp; incr body wt & anterior pituitary wt at sac in 10 mg grp	persistent vaginal estrous likely due to a hypothalamic rather than ovarian defect	Gellert 1978
Aroclor 1242	1 mg; pnd 2-3; sc	Rat Sprague Dawley	no significant effect on VO, estrus cycles, ovulation, body or organ wt		Gellert 1978
Aroclor 1254	1 mg; pnd 2-3; sc	Rat Sprague Dawley	no significant effect on VO, estrus cycles, & ovulation; decr adrenal wt		Gellert 1978
Aroclor 1260	1 mg; pnd 2-3; sc	Rat Sprague Dawley	no significant effect on VO, estrus cycles, ovulation, body or organ wt		Gellert 1978
Aroclor 1221	1, 10, 100, 1000 mg/kg; 1x; sc	Rat Sprague Dawley	incr uterine wt at 1000 mg/kg		Gellert 1978
Aroclor 1242	1, 10, 100, 1000 mg/kg; 1x; sc	Rat Sprague Dawley	no significant effect on uterine growth		Gellert 1978
Aroclor 1254	1, 10, 100 mg/kg; 1x; sc	Rat Sprague Dawley	no significant effect on uterine growth		Gellert 1978
Aroclor 1260	1, 10, 100 mg/kg; 1x; sc	Rat Sprague Dawley	no significant effect on uterine growth		Gellert 1978

Table 5. Selected Studies of Estrogen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
Tween 80	0.1 ml of 1, 5, or 10% solution; pnd 4-7; ip	Rat strain not specified	accelerated VO , prolonged estrous cycle, shortened diestrous, early onset of pro-estrous, prolonged end of pro-estrous, & prolonged beginning of meta-estrous in all grps; ovarian cavities in 10% grp; decr ovary wt in all grps; decr uterus wt in 5% grp; incr adrenal wt in 1% grp; squamous cell metaplasia of uterine epithelial lining & cytological changes; lack of corpora lutea & presence of degenerative follicles in ovaries		Gajdov et al. 1993
diethylstilbestrol	0.0037, 0.037, 0.37 mg/kg/d; pnd 2,4,6,8,10, 12; sc	Rat Wistar	dose-dependent decr in testis wt, AQP-1 immunoexpression, epithelial cell height & efferent duct lumen distention & rete testis at all time points		Fisher et al. 1999
diethylstilbestrol	1, 10, 100 µg/kg/d gd 6 - weaning; sc	1, 10, 100 µg/kg/d gd 6 - weaning sc	dose-related incr post-implantation loss; decr mean litter size in high dose grp; disrupted spermatogenesis in male offspring causing disorganized & immature tubules & Leydig cell hyperplasia		Kledal et al. 2000
diethylstilbestrol	0.01, 0.1 µg/kg/d gd 17 & 19; sc	Rat Donryu	in 0.1 mg/kg grp decr mean litter size & pregnancy length, disrupted estrous cycle; incr hypoplasia of oviduct, cystic dilation of uterus & small size of uterine cervix in female offspring; incr atrophy in ovary of 001 and 0.01 mg/kg offspring; dose-dependent incr in endometrial adenocarcinomas; incr mucification of vagina in treated offspring		Kitamura et al. 1999
ethinyl estradiol	0.37 mg/kg/d; pnd 2, 4, 6, 8, 10, 12; sc	Rat Wistar	decr testis wt at days 18,-75; dose-dependent decr in immunoreactive AQP-1, distension of efferent duct lumen at day 18-75; decr epithelial cell height at day 18; gross distention of rete testis at day 18		Fisher et al. 1999

Table 5. Selected Studies of Estrogen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
octylphenol	150 mg/kg; pnd 2-12; sc	Rat Wistar	incr testis wt at day 18; decr efferent ducts epithelial cell height at day 18 & 25; slight decr AQP-1 immunostaining, slight incr in efferent duct lumen size at day 18 & 25; no effect on rete testis morphology	suggests due to direct effect	Fisher et al. 1999
butylparabens	approx. 2 mg/kg/d; pnd 2-18; sc	Rat Wistar	no effect testis wt; AQP-1 immunoexpression, efferent duct or rete testes morphology, or efferent duct epithelial cell height		Fisher et al. 1999
genistein	appro 4 mg/kg/d; pnd 2-18; sc	Rat Wistar	decr epithelial cell height of efferent ducts at day 18 &/or 25; incr testis wt as adults; no effect on rete testis morphology	suggests due to direct effect	Fisher et al. 1999
tamoxifen	approx. 2 mg/kg; pnd 2-16; sc	Rat Wistar	AQP-1 changes, efferent duct lumen distention, decr epithelial cell height at day 18		Fisher et al. 1999
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	1 µg/kg; gd 11, 15, or 18; po	Rat Holtzman	incr cleft clitoris & vaginal thread for all exposure days; no change in VO, ovarian morphology, or reduction in no. of primordial follicles		Flaws et al. 1997
endosulfan	1.5, 3.0 mg/kg; gd 15 - pnd 22; po	Rat Wistar	incr relative testes wt & decr daily sperm production at puberty for 1.5 & 30. mg/kg; incr relative testes wt & decr daily sperm production at adulthood for 3.0 mg/kg; decr seminiferous tubule w/ complete spermatogenesis at puberty for 1.5 & 3.0 mg/kg		Dalsenter et al. 1999
p-nonylphenol	100, 250, 400 mg/kg; gd 7 - weaning plus 10 wks; 7d/wk; po	Sprague Dawley	no litters at 400 mg/kg; decr body mass, testicular mass, & seminiferous tubule diameter & seminiferous epithelium thickness at 100 & 250 mg/kg; decr testes ratio, epididymal mass, & sperm count at 250 mg/kg		de Jager et al. 1999
bisphenol A	200 mg/kg/d; 2d; ovarianectomized; sc	Rat Sprague Dawley	incr uterine wt; induced lordosis behavior		Gray et al. 1999
bisphenol A (BPA)	400, 600, 800 mg/kg; 1x/d; 3 d; po	Rat Alpk:AP	incr uterine wt.; premature VO		Ashby and Tinwell, 1998

Table 5. Selected Studies of Estrogen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	bisphenol A (BPA)	400, 600, 800 mg/kg; 1x/d; 3 d; sc	Rat Alpk:AP	incr uterine wt.; premature VO (at 600 & 800 mg/kg)		Ashby and Tinwell, 1998
2 3	bisphenol A	37 mg/kg/d; pnd 2-12; sc	Rat Wistar	decr epithelial cell height of efferent ducts at day 18; decr testis wt day 35; no gross changes in level of AQP-1 immunostaining or efferent duct or rete testis morphology	suggests due to direct effect	Fisher et al. 1999
4	bisphenol A (BPA)	150 mg/kg; 20d (35-36 days old); po	Rat Alpk:APfSD	decr. liver wt		Ashby and Lefevre, 2000
5 6	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	1 µg/kg; gd 15; po	Rat Holtzman	incr relative ovary wt; incr ER mRNA in hypothalamus, uterus, & ovary; decr ER mRNA in pituitary; incr DNA-binding in uterus, decr DNA-binding in hypothalamus; decr circulating estrogen concentration	actions of TCDD in this model are very complex & should not necessarily be defined as purely anti-estrogen or estrogenic	Chaffin et al. 1996a
7	methoxychlor (MXC)	0, 5, 50, 150 mg/kg; gd 17 - pnd 7 (dams) pnd 7 - pnd 21 or 42 (pups); po	Rat Sprague Dawley	dose-dependent amounts of MXC & metabolites in milk & plasma of dams & pups; 17% decr litter size at 150 mg/kg; accelerated VO all grps; delayed preputial separation at 50 & 150 mg/kg; incr excitability in 150 mg/kg males; decr antibody plaque-forming cell response in males; disrupted estrous cyclicity, pregnancy & delivery rates at 50 & 150 mg/kg; decr uterine wts in all treated females; males at 150 mg/kg impregnated fewer untreated females; decr epididymal sperm count at 150 mg/kg; decr testes wt at 50 & 150 mg/kg; decr epididymal, seminal vesicle, & prostate wt at 150 mg/kg; uterine dysplasia, decr mammary alveolar development & estrous levels of FSH in all treated females; decr estrous progesterone at 50 & 150 mg/kg	cites of action both central & peripheral	Chapin et al. 1997

Table 5. Selected Studies of Estrogen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
raloxifen hydrochloride	0, 0.1, 1, 10 mg/kg/d; gd 6 - pd 20; po	Rat Sprague Dawley	decr. maternal body wt. & food consumption; progressive dose-related decr in litter size & pup body weight during lactation; accelerated negative geotaxis & incisor eruption (1 & 10 mg/kg); delayed eye opening (10 mg/kg); dose-related decr in spleen cellularity & thymus weights; age & sex specific postweaing body weights & growth parameters & pituitary hormone content effects; advanced vaginal patency; disrupted estrous cycle at 12-14 wks & poor mating & fertility indices (10 mg/kg); vaginal mucification uterine hypoplasia	decr maternal body wt may act as an estrogen agonist at those central hypothalamic areas that regulate consumatory behavior; repro & developmental effect profile suggest estrogen antagonist activity	Buelke-Sam et al. 1998
ethinyl estradiol	0.02, 0.2 mg/kg; gd 11-17; po	Mouse ICR	no live offspring at 0.2 mg/kg; decr testosterone in testes of aged & fetal offspring at 0.02 mg/kg; decr estrogen in testes of fetuses & incr estrogen in aged offspring at 0.02 mg/kg; decr testosterone & estrogen in testes of fetuses at 0.2 mg/kg; incr seminiferous tubule atrophy in 83%, Leydig's cell hyperplasia in 67%, precancerous changes in epididymis in 17% aged male offspring of 0.02 mg/kg grp;	effects may be due to decr testosterone & it's conversion to estradio-17β in fetal testes at critical periods induced by ethinyl estradiol	Yasuda et al. 1988
tamoxifen	20 μg; gd 15-20; sc	Rat Sprague Dawley	in female offspring delayed eye opening & age at vaginal opening; decr uterine wt; incr incidence mammary tumors that continued to grow in offspring treated with 7,12-dimethylbenz[a}antracene on pnd 45; tumors less differentiated and more aggressive		Hilakivi-Clarke et al. 2000
[^{3}H]ethinyloestradiol	15-25 μCi; gd 10.5, 14.5, or 18.5; po	Rat Sprague Dawley	radioactivity in placentas of dams from each day and in fetal liver, gonads & external genitalia on gd 14.5 and 18.5		Varma and Bloch 1987
[^{3}H]mderoxyprogesterone acetate	up to 20 μCi; gd 14.5; po	Rat Sprague Dawley	radioactivity in placenta & fetal tissues		Varma and Bloch 1987
medroxyprogesterone-acetate	2, 20 mg/kg; gd 14.5-19.5; po	Rat Sprague Dawley	no change in testes function or development		Varma and Bloch 1987

Table 5. Selected Studies of Estrogen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	norethisterone	2, 20 mg/kg; gd 14.5-19.5; po	Rat Sprague Dawley	no change in testes function or develpment		Varma and Bloch 1987
2	oestrogen mestranol	0.01, 0.1 mg/kg; gd 14.5-19.5; po	Rat Sprague Dawley	in 0.1 mg/kg grp 35-70% decr LH-stimulated testosterone synthesis in fetal and adult testes, decr sera testosterone		Varma and Bloch 1987
3	butyl benzyl phthalate (BBP)	500 mg/kg; 14d (22-23 days old); po	Rat Alpk:APfSD	incr. liver wt		Ashby and Lefevre, 2000
4	butly benzyl phthalate (BBP)	500 mg/kg; 20d (35-36 days old); po	Rat Alpk:APfSD	incr. liver & combined kidney wt.		Ashby and Lefevre, 2000
5	methoxychlor	50 mg/kg; 20d (35-36 days old); po	Rat Alpk:APfSD	decr. final body wt, decr. prostate wt.		Ashby and Lefevre, 2000
6	methoxychlor	100 mg/kg; 20 d (35-36 days old); po	Rat Alpk:APfSD	decr. final body wt.decr liver & combined seminal vesicles wt.		Ashby and Lefevre, 2000
7	diethylstilbestrol (DES)	40 µg; 14d (22-23 days old); po	Rat Alpk:APfSD	decr. combined testes, combined epididymides, combined seminal vesicles wts		Ashby and Lefevre, 2000
8	diethylstilbestrol (DES)	40 µg; 14d (35-36 days old); po	Rat Alpk:APfSD	decr final body wt;decr. combined testes, combined epididymides, combined seminal vesicles & prostate wts		Ashby and Lefevre, 2000
9	diethylstilbestrol (DES)	40 µg; 20d (35-36 days old); po	Rat Alpk:APfSD	decr final body wt; decr combined seminal vesicles & prostate wts; delayed prepuce separation		Ashby and Lefevre, 2000
10	diethylstilbestrol (DES)	40 µg; 14d (22-23 days old); po	Rat Alpk:APfSD	decr combined testes, combined epididymides, combined seminal vesicles, & prostate wts; delayed prepuce separation		Ashby and Lefevre, 2000
11	diethylstilbestrol (DES)	40 µg; 20d (35-36 days old); po	Rat Alpk:APfSD	decr final body wt; decr liver, combined testes, combined seminal vesicles, & prostate wt		Ashby and Lefevre, 2000
12	diethylstilbestrol (DES)	40 µg; 14d (22-23 days old) 20 day recovery; po	Rat Alpk:APfSD	decr final body wt; decr combined testes, combined epididymides & combined seminal vesicles		Ashby and Lefevre, 2000
13	diethylstilbestrol (DES)	40 µg; 34 d (35-36 days old); po	Rat Alpk:APfSD	decr final body wt; decr liver, combined testes, combined epididymides, seminal vesicles, & prostate wts; delayed PPS		Ashby and Lefevre, 2000

Table 5. Selected Studies of Estrogen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
fenitrothion	15 mg/kg; 14 d (35-36 days old); po	Rat Alpk:APfSD	decr final body wt		Ashby and Lefevre, 2000
bisphenol A	0.2, 2, 20, 200 ug/kg/d; gd 11-17; oral	Mice CF-1	no significant effects on testes histopathology, daily sperm production, or sperm count, or on prostate, preputial gland, seminal vesicle, or epididymis weights		Cagen et al. 1999a
bisphenol A (BPA)	0.1, 54 mg/kg/d; 11 days; sc	Rat Noble	estrogenic-like stimulation of growth and differentiation of the mammary gland ductal and glandular components		Colerangle and Roy 1997
methoxychlor	25, 50, 100, 200 mg/kg/d; weaning, through puberty & gestation, to day 15 of lactation; gavage	Rat Long-Evans	decr food consumption and growth, delayed puberty, reduced sex accessory gland weight, testicular testosterone production ex vivo, epididymal size, and epididymal sperm numbers, and stimulateed mating behavior		Gray et al. 1989
bisphenol A	2, 20 ppb; gd 11-17; oral	Mice CF-1	incr size of preputial glands, reduced size of epididymides at 2 ppb; decr efficiency of sperm production at 20 ppb		vom Saal et al. 1998
octylphenol	2, 20 ppb; gd 11-17; oral	Mice CF-1	reduced daily sperm production and efficiency of sperm production at 2 ppb		vom Saal et al. 1998
octylphenol	0.2, 20, 200, 2000 ppm; oral	Rat CD	decr body weights in adults and during the latter portion of lactation in offspring, minor body weight-related delays in acquisition of vaginal opening and preputial separation; no effects on reproductive parameters		Tyl et al. 1999
butyl benzyl phthalate	1000 µg/L (ave. 182.6 µg/kg/d) gd 1- pnd 21 drinkig water	Rat AP_fSD	incr anogenital distance in males; accelerated VO		Ashby et al. 1997a
diethylstilbistrol	50 µg/L (ave. 8.6 µg/kg/d) gd 1- pnd 21 drinkig water	Rat AP_fSD	decr anogenital distance in males & females; accelerated VO; delayed PPS; decr testis, & epididymis wt pnd 90 & 137; decr seminal vesicle wt pnd 90; decr cauda & testis sperm pnd 90 & 137		Ashby et al. 1997a

Table 5. Selected Studies of Estrogen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	bisphenol A	2, 20 µg/ kg gd 11-17 oral not gavage	Mouse CF1	no significant effects on offspring development including prostate wt, sperm efficiency, sexual maturation, reproductive tissue wts		Ashby et al. 1999
2	diethystibistrol	0.2 µg/kg gd 11-17 oral not gavage	Mouse CF1	no significant effects on offspring development including prostate wt, sperm efficiency, sexual maturation, reproductive tissue wts		Ashby et al. 1999
3	17β-estradiol	0.05, 2.5, 10, 50 ppm 90 d (approx. pnd 49-139) in diet	Rad CD BR	in P1 animals dose-dependent incr serum estradiol (E2) in 2.5, 10, & 50 ppm grps; dose-dependent decr serum progesterone (P4) on day 90 w/ no corpora lutea & ovarian atrophy in 2.5 ppm & greater; decr LH at all time points for 10 & 50 ppm grps and 2.5 ppm grp on day 90; incr serum prolactin in 50 ppm grp on day 90; no F1 offsping produced at 10 & 50 ppm; decr number of estrous cycles, incr mean cycle length, decr number of normally cycling rats in 2.5 ppm and up groups; in F1 offspring incr serum E2 at 2.5 ppm; decr number of estrous cycles incr mean cycle length, decr number of normally cycling rats in 2.5 ppm grp		Biegel et al. 1998a
4	17β-estradiol	0.05, 2.5, 10, 50 ppm 90 d (approx. pnd 49-139) in diet	Rat CD BR	decr corpora lutea & large antral follicles at 2.5 ppm & up; at 10 & 50 ppm diffuse hyperplasia of pituitary, feminization of male mammary glands, mammary gland hyperplasia in females, incr cystic folliclesn in ovary, hypertrophy of endometrium & endometrial glands in uterus, degeneration of seminiferous epithelium, testes and accessory gland atrophy; no litters produced in 10 or 50 ppm grp		Biegel et al. 1998b

Table 5. Selected Studies of Estrogen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	17β-estradiol	0.05, 2.5, 10, 50 ppm F1 in diet	Rat CD BR	delayed PPS in 2.5 ppm grp; accelerated VO in 0.05 and 2.5 ppm grps; incr pituitary wt in 2.5 ppm grp; diffuse pituitary hyerplasia, mammary gland hyperplasia, decr large antral follicles, hypertorpy of endometrium & endometrial glands in ovaries in uterus, incr estrous, thickening of mucosa of prosetrual vagina in 2.5 ppm grp females; decr testes, epididymal, & accessory gland wt in 2.5 ppm males		Biegel et al. 1998b
2	methoxychlor	0.3, 1.7, 6.7 μM in vitro	Rat Spague-Dawley	suppressed [^{3}H]estradiol-17β binding to 8S estrogen receptor	inhibition of binding competitive and not caused by receptor destruction	Bulgar et al. 1978
3	base-washed methoxychlor	0.3, 1.7, 6.7 μM in vitro	Rat Spague-Dawley	suppressed [^{3}H]estradiol-17β binding on sucrose gradients at concentrations as low as 3.6 ppm		Bulgar et al. 1978
4 5	2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane	0.3, 1.7, 6.7 μM in vitro	Rat Spague-Dawley	suppressed [^{3}H]estradiol-17β binding to 8S estrogen receptor	effect altered the affinity of binding receptor in a competitive manner	Bulgar et al. 1978
6	diethylstilbestrol	0.1 ppm 7d/wk 10 wk (premating - lactation) drinking water	Rat Han-Wistar	decr duration of gestation; decr no. of pups delivered and no. of live pups; no effects on male offspring reproductive organs		Cagen et al. 1999b
7	bisphenol A	0.01, 0.1, 1.0, 10.0 ppm 7 d/wk 10 wk (premating - lactation) drinking water	Rat Han-Wistar	no effects on testes, prostate, and preputial gland wt; no effect on sperm count, daily sperm production, or testes histopathology		Cagen et al. 1999b
8	4-octylphenol	10, 100, 1000 μg/L pnd 1-22 drinking water	Rat Wistar	decr ave testis wt at 1000 μg/L; decr relative testis wt at 100 & 1000 μg/L		Sharpe et al. 1995
9	diethylstibestrol	100 μg/L pnd 1-22 drinking water	Rat Wistar	decr ave & relative testis wt		Sharpe et al. 1995

Table 5. Selected Studies of Estrogen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
4-octylphenol	100, 1000 µg/L 2 wks prior to mating - pnd 22 drinking water	Rat Wistar	decr mean testicular wt at 100 (nonculled litters) &1000 µg/L; decr relative testis wt at 100 & 1000 µg/L (1000 µg/L only for nonculled litters); decr ventral prostate wt (nonculled litters) at 100 &1000 µg/L; decr daily sperm production at 1000 µg/L		Sharpe et al. 1995
butyl benzyl phthalate	1000 µg/L 2 wks prior to mating - pnd 22 drinking water	Rat Wistar	decr mean & relative testis wt; decr daily sperm production		Sharpe et al. 1995
diethylstibestrol	100 µg/L 2 wks prior to mating - pnd 22 drinking water	Rat Wistar	decr mean & relative testis wt, decr ventral prostate wt (culled litters only); decr daily sperm production		Sharpe et al. 1995
octylphenol polyethyoxylate (weak)	1000 µg/L 2 wks prior to mating - pnd 22 drinking water	Rat Wistar	decr mean & relative testis wt	effect attributed to OPP's metabolism in vivo to OP	Sharpe et al. 1995

Table 5. Selected Studies of Estrogen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	bisphenol A	3.2, 32, 320 mg/kg/d gd 11-pnd 20 po	Rat Sprague-Dawley	no effect on female pubertal development; no effect on volume of sexually dimorphic nucleus of preoptic area, estrous cyclicity, sexual behavior or male reproductive organ wts in F1 offspring		Kwon et al. 2000
2	diethylstilbestrol	15 μg/kg/d gd 11-pnd 20 po	Rat Sprague-Dawley	incr volume of sexually dimorphic nucleus of preoptic area in females and affected estrous cyclicity		Kwon et al. 2000
3	octylpheonl	2, 20 μg/kg gd 11-17 oral not gavage	Mouse CF-1	no effect on prostate wt		Nagel et al. 1997
4						

5

γ-GT = γ-glutamyl dehydrogenase
AR = androgen receptor
CNS = central nervous system
d = days
decr = decreased
FSH = follicle stimulating hormone
gd = gestational day
grp = grp
GSH = glutathione
hCG = human chorionic gonadotropic
inc = increased
ld = lactation day
LDH = lactate dehydrogenase
LH = luteinizing hormone
pc = post coitum
pd = postpartum day
pnd = postnatal day
po = gavage
PPS = preputial separation
sc = subcutaneous
SDH = sorbitol dehydrogenase
TSH = thyroid stimulating hormone
VO = vaginal opening
w/o = without
w/ = with
wks = weeks
wt = weight

15

16

Table 6. Selected Studies of Androgen Modulators

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
diethylhexyl phthalate (DEHP)	750 mg/kg/d; trans gen; gd 14-pnd 3; 1 x/d; po	Rat Sprague Dawley	in F1 male offspring decr pup wt on d 2, anogenital distance. seminal vesicle, ventral prostate, epididymides, cauda epididymis, testes, glans penis and levator ani-bulbocavernosus muscle wt; incr nipple retention and no. of nipples		Gray et al. 1999c
polychlorinated biphenyl congener No. 169	1.8 mg/kg; trans gen; 1x, gd 8; po	Rat Long Evans	F1 offspring incr % eye open pnd 14,15, 16; in males delayed puberty; in females incr % transient and permanent vaginal thread, incr % w/ mild hypospadias, cleft phallus, incr size urethral slit, decr distance urethral to vaginal opening		Gray et al. 1999c
polychlorinated biphenyl congener No. 169	1.8 mg/kg; trans gen; 1x, gd 8; po	Rat Long Evans	F1 male offspring pnd 65 decr ventral prostate, seminal vesicles, testes, epididymides, cauda epididymides, epididymal wt; decr epididymal, testis, and caudal sperm count; same effects at pnd 169 plus acute prostatitis in 70% & decr ejaculated sperm count		Gray et al. 1999c
ethane dimethane sulphonate (EDS)	50 mg/kg/d; trans gen; gd 14-21; po	Rat Long Evans	decr mat. wt gd 14; decr pup viability; 45% decr pup wt' 13.1% decr anogenital distance in males;		Gray et al. 1999c
ethane dimethane sulphonate (EDS)	50 mg/kg/d; trans gen; gd 14-21; po	Rat Long Evans	decr mat. wt gd 14; decr pup viability; 45% decr pup wt' 13.1% decr anogenital distance in males;		Gray et al. 1999c
linuron (L)	10, 20, 40 mg/kg/d; multi gen; pnd 21-lactation; 1x/d; po	Rat Long Evans	decr seminal vesicle and cauda epididymal wt & delayed PPS in F0 males at 40 mg/kg; decr no. pups, testes wt, spermatid no., & epididymal wt in F1 males in 40 mg/kg grp		Gray et al. 1999c
linuron (L)	100 mg/kg/d; trans gen; gd 14-18; 1x/d; po	Rat Sprague Dawley	decr pup wt pnd 0; in male pups decr anogenital distance, incr nipple retention, epispadias in 1/13, epididymal and testicular malformations including agenesis and atrophy; decr seminal vesicle, ventral prostate, levator ain/bulbocavernosus muscle, and epididymide wt; decr testes wt		Gray et al. 1999c
p,p'-DDE	100 mg/kg/d; trans gen; gd 14-18; po	Rat Long Evans	in F1 male offspring incr % w/ areolas & nipple retention; decr ventral prostate wt; incr atrophy		Gray et al. 1999c

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
p,p'-DDE	100 mg/kg/d; trans gen; gd 14- 18; po	Rat Sprague Dawley	in F1 male offspring decr anogenital distance; decr glans penis, cauda epididymis, ventral prostate and levator ani-bulbocavernosus muscle wt; incr % w/ areolas, nipple retention		Gray et al. 1999c
ketoconazole	12.5, 25, 50 mg/kg/d; trans gen; gd 14-pnd 3;	Rat Long Evans	F0 delayed delivery and decr no dams w/ viable litters at pnd 4 in 25 and 50 mg/kg, decr litter size pond 2 in 25 mg/kg grp ; in F1 male offspring decr testes, seminal vesicle, & epididymal wt in 50 mg/kg grp	suggest inhibits synthesis of estradiol near term and possible inhibiting aromatase activity	Gray et al. 1999c
procymidone (P)	100 mg/kg/d; trans gen; gd 14-pnd 3; po	Rat Long Evans	in F1 male offspring incr anogenital distance; incr nipple retention; decr ventral prostate, levator ani-bulbocavernosus muscle, seminal vesicle wt; incr hypospadias, chronic active prostatis or focal mononuclear cell infiltration, chronic active vesiculitis		Gray et al. 1999c
chlozolinate	100 mg/kg/d; transgen; gd 14- pnd 3; po	Rat Sprague Dawley	no demasculinizing or feminizing effects on F1 male offspring		Gray et al. 1999c
iprodione	100 mg/kg/d; transgen; gd 14- pnd 3; po	Rat Sprague Dawley	no demasculinizing or feminizing effects on F1 male offspring		Gray et al. 1999c
dibutyl phthalate (DBP)	250, 500, 1000 mg/kg/d; multi gen; pnd 21-lactation; 1x/d; po	Rat Long Evans	delayed PPS in male F0 at all doses; decr fertility in F0 males and females at 500 and 1000 mg/kg; in F1 males & females in 250 and 500 mg/kg grp low hypospadias, testicular nondescent, anophthalmia, uterus unicornous, renal agenesis, decr cauda epididymal sperm		Gray et al. 1999c
dibutyl phthalate (DBP)	500 mg/kg/d; trans gen; gd 16-19; 1x/d; po	Rat Long Evans	in F1 male offspring decr anogenital distance; incr nipple retention; decr seminal vesicle, ventral prostate, & levator ani-bulbocavernosus wt; incr no. of nipples		Gray et al. 1999c
dibutyl phthalate (DBP)	500 mg/kg/d; trans gen gd 14 - pnd 3; 1x/d; po	Rat Sprague Dawley	in F1 male offspring decr anogenital distance; incr nipple retention; decr ventral prostate, epididymides, cauda epididymis, testes, glans penis, levator ani-bulbocavernosus muscle wt		Gray et al. 1999c

Table 6. Selected Studies of Androgen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	dibutyl phthalate (DBP)	200, 400 mg/kg/d; 2d ovariectomized; sc	Rat Sprague Dawley	no incr uterine wt; did not induce lordosis behavior		Gray et al. 1999c
2	dibutyl phthalate (DBP)	1000 mg/kg/d; 2d ovarianectomized; po	Rat Sprague Dawley	no incr uterine wt; did not induce lordosis behavior		Gray et al. 1999c
3	dibutyl phthalate (DBP)	250, 500, or 750 mg/kg/day gd 3-20; po	Rat CD	decreased number of pups per litter at high dose, decreasd AGD at mid and high dose, absent or underdeveloped epididymis at all doses (pnd 100) associated with testicular atrophy and germ cell loss; hypospadias, absence of prostate and seminal vesicles , as well as small testis and seminal vesicles	antiandrogen	Mylchreest et al., 1998
4	vinclozolin	100, 200 mg/kg/d; gd 14-pnd 3; po	Rat Long Evans	decr anogenital distance and nipple retention in treated male offspring at 2 wks; decr attained intromissions failure to ejaculate or w/o sperm in treated male offspring at puberty; cleft phallus with hypospadias in treated male offspring & reproductive malformations; decr neonatal anogenital distance in female offspring		Gray et al. 1994
5	dibutyl phthalate	250, 500, 1000 mg/kg/d; pnd 21-approx. 150 (females); route not specified	Rat strain not specified	decr no. impl sites in 500 & 1000 mg/kg grps; decr litter size in 1000 mg/kg grp		Gray et al. 1988
6	dibutyl phthalate	250, 500, 1000 mg/kg/d; pnd 21-100 (males); route not specified;	Rat strain not specified	decr testis wt, caudal epididymal wt, caput epididymis wt in 500 & 1000 infertile & 500 fertile grps; decr seminiferous tubule fluid wt, serum ABP, caudal epididymal sperm count, testicular sperm head count, & incr serum LH &FSH in 500 & 1000 infertile grps; incr IF testosterone, hCG testosterone, pituitary LH, & decr epididymal ABP in 1000 infertile grp		Gray et al. 1988
7	di-n-butyl phthalate	1000, 1500 mg/kg; gd 12-14; po	Rat Wistar	decr maternal body wt gain/food consumption; incr resorptions; decr fetus wts; incr fetuses w/ undescended testes at 1500 mg/kg; decr anogenital distance for male fetuses	effects mediated by the metabolite monobutyl phthalate	Ema et al. 2000

Table 6. Selected Studies of Androgen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	di-n-butyl phthalate	1000, 1500 mg/kg; gd 18-20; po	Rat Wistar	decr maternal body wt gain/food consumption; decr fetus wts; decr anogenital distance for male fetuses	effects mediated by the metabolite monobutyl phthalate	Ema et al. 2000
2	di-n-butyl phthalate	500, 1000, 1500 mg/kg; gd 15-17; po	Rat Wistar	decr maternal body wt gain/food consumption; incr resorptions; decr fetus wts as 1500 mg/kg; incr fetuses w/ undescended testes; decr anogenital distance for male fetuses	effects mediated by the metabolite monobutyl phthalate	Ema et al. 2000
3 4	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	0.2 or 1.0 µg/kg; gd 15; po	Rat Sprague Dawley	decr body wt gain in both TCDD grps; decr litter size at 1.0 µg/kg; at 30 d old decr 17-hydroxylase activity, lower caput-corpus epididymal wt in 1.0 µg/kg offspring; at 45 d old incr 3β-HSD, 17β-HSD, & 5α-reductase activity in 1.0 µg/kg offspring; decr serum androgens at 45 d old; decr 17β-HSD activity at 90 d old		Cooke et al. 1998
5 6	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	0.7 µg/kg; gd 15; po	Rat Holtzman	decr anogenital distance & crown-rump length; delayed testes descent & PPS; decr paired testes weight; decr responsiveness of ventral prostate weight & protein content to testosterone	inhibition of imprinting of ventral prostate weight & protein	Bjerke et al. 1994a
7	fenarimol	50, 130, 350 ppm; multi gen F0; approx. pnd 21-90; in diet	Rat Wistar	dose-dependent decr. no. pregnant dams; decr. fertility at 2nd and 3rd mating at 130 (2nd mating only) & 350 ppm	inhibits conversion of testosterone to estrogens (CNS)	Hirsch et al. 1986
8	fenarimol	50, 130, 350 ppm; multi gen F1b; approx. pnd 21-79 (58d); in diet	Rat Wistar	decr no. fertile matings at 130 ppm & no pregnancies at 350 ppm in 1st & 2nd matings; effects more pronounced w/ increasing age and were only partially reversible	inhibits conversion of testosterone to estrogens (CNS)	Hirsch et al. 1986
9	fenarimol	F0: 35 mg/kg/d; 4 wk before mating through 1 mo post mating (males); 4 wk before mating through 2 wk lactation (females); po	Rat Wistar	35% decr no. pregnancies from males in 35 mg/kg grp w/o anatomical abnormalities; male-specific reproductive effect	inhibits conversion of testosterone to estrogens (CNS)	Hirsch et al. 1986
10	fenarimol	350 ppm; gd 0-14, 15, 16 17, or 18; in diet	Rat Wistar	25% decr plasma ^{14}C after 6 hrs; decr fetal ^{14}C ; limited placental transport;	inhibits conversion of testosterone to estrogens (CNS)	Hirsch et al. 1986

Table 6. Selected Studies of Androgen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	fenarimol	F0: 350 ppm; pnd 0 - end of study; in diet; then 2.1 mg/kg ^{14}C- fenarimol pnd 5; po	Rat Wistar	quantity of radioactivity in milk greater than in plasma	inhibits conversion of testosterone to estrogens (CNS)	Hirsch et al. 1986
2	fenarimol	350 ppm; pnd 0 - end of study; in diet; then 2.1 mg/kg ^{14}C- fenarimol; pnd 5; po	Rat Wistar	incr brain wt and protein ; conc of radioactivity in hypothalamus incr rapidly and decr slowly	inhibits conversion of testosterone to estrogens (CNS)	Hirsch et al. 1986
3	fenarimol	17.5, 35, or 70 mg/kg/day F0 from weaning through lactation of F1; po	Rat Wistar	Males exhibited altered mating behavior	inhibits conversion of testosterone to estrogens (CNS)	Hirsch et al. 1987
4 5	2,3,7,8-tetrachlorodibenzo-p-dioxin	1.0 μg/kg; gd 15; po	Rat Long Evans	decr male pup total seminal vesicle wt on pnd 32; decr body & seminal vesicle & total seminal vesicle wt on pnd 49, 63, 120; decr seminal vesicle fluid on pnd 49; decr epithelium w/ decr branching & differentiation characterized by rounded nuclei & less cytoplasm at pnd 32; immunolocalization of proliferating nuclear antigen in basal epithelial & luminal cells of treated seminal vesicle		Hamm et al. 2000
6	flutamide	0.1, 1, 10 mg/kg/d; gd 6-weaning; sc	Mouse SV	dose-related incr post-implantation loss; decr mean litter size in high dose grp		Kledal et al. 2000
7 8	p,p'-DDE	1, 10, 50, 100, 200 mg/kg gd 14-18; po	Rat Holtzman	in 200 mg/kg grp decr wts all dam & male offspring & incr fetal mortality; decr anogenital distance to crown rump length ratio in 50 mg/kg grp & above on pnd 1 and 4 (200 mg/kg grp only); dose dependent incr nipple retention pnd 13; delayed preputial separation in 200 mg/kg grp; decr ventral prostate wt on pnd 21 in 50 mg/kg grp & above; decr dorsolateral prostate w at 200 mg/kg on pnd 21 & all treated grps on pnd 32	competitive antagonist	Loeffler and Peterson 1999

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
2,3,7,8-tetrachlorodibenzo-p-dioxin	0.25 μg/kg; gd 15; po	Rat Holtzman	in male offspring decr ventral prostate wt pnd 21 & 32; decr dorsolateral prostate wt on pnd 21; decr cauda epididymal sperm no.; on pnd 21 hyperplastic epithelium of prostatic ducts, dysplastic epithelial nuclei, convoluted prostatic ducts		Loeffler and Peterson 1999
p,p'-DDE	100 mg/kg; gd 14-18; po	Rat Holtzman	in male offspring, decr ventral prostate wt pnd 21 & 32; decr dorsolateral prostate wt on pnd 21; decr cauda epididymal sperm no.; retained nipples on pnd 13; decr androgen receptor labeling	androgen antagonist	Loeffler and Peterson 1999
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) & p,p'-DDE	0.25 μg/kg (TCDD), 100 mg/kg (p,p'-DDE) gd 15 (TCDD) & gd 14-18 (p,p'-DDE); po	Rat Holtzman	in male offspring, decr ventral prostate wt pnd 21 & 32; decr dorsolateral prostate wt on pnd 21:retained nipples on pnd 13; on pnd 21 hyperplastic epithelium of prostatic ducts, dysplastic epithelial nuclei, convoluted prostatic ducts		Loeffler and Peterson 1999
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) -	1.0 μg/kg; gd 15; po	Rat Holtzman	delayed PPS, decr plasma testosterone, decr daily sperm production & epididymal sperm reserves; feminized sexual behavior		Bjerke and Peterson 1994
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	0.7 μg/kg; gd 15; po	Rat Holtzman	incr intromission latencies; incr no. intromissions prior to ejaculation; incr freq & intensity of lordotic behavior after castration & ovarian steroids	partial demasculinization & feminization not associated w/ effect on sexual differentiation of estrogen receptor system or volume of sexually dimorphic brain nuclei	Bjerke et al. 1994b
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	15, 30, or 60 microg/kg 1x on gd 14; po	Mouse ICR	Offspring evaluated on pnd 44, 65, 114/128. No effect on maternal or offspring body weight; ventral prostate weight, coagulating gland weight, eye opening (males), and thymus and pituitary weight (males) were affected at lowest dose. Decreased offspring uterine weight observed at highest maternal dose.		Theobald and Peterson, 1997

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	1.0 micrograms/kg; gd 15; po	Rat Holtzman	decreased daily sperm production per testis, corpus and cauda epididymis sperm numbers, vas deferens sperm number, and ejaculated sperm number on pnd 92-93.		Sommer et al., 1996
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	1.0 micrograms/kg; gd 15; po	Rat Holtzman	decreased AGD, delayed PPS, decreased ventral and dorso-lateral prostate weight		Roman et al., 1995
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	1.0 micrograms/kg; gd 15; po	Rat Holtzman	Impaired prostatic epithelial budding on gd 20; decreased ventral prostatic cell proiferation on pnd 1; delayed differentiation of pericordal smooth muscle cells and luminal epithelial cells; alterations in ventral prostate histological arrangement of cells (disorganized) and androgen receptor distribution on pnd 21 and 32.	Decreased very early prostatic epithelial growth, delayed cytodifferentiation, alterations in epithelial and stromal cell histological arrangement and spatial distribution of androgen receptor expression.	Roman et al., 1998
2,3,7,8-tetrachlorodibenzo-p-dioxin	1.0 μg/kg; gd 15; po	Rat Holtzman	7-22% decr body wt for male neonates pnd 0-5; decr survival pnd 19-21; decr plasma testosterone in male fetuses throughout gestation; decr surge of plasma testosterone after birth; decr anogenital distance		Mably et al. 1992a
2,3,7,8-tetrachlorodibenzo-p-dioxin	0.064, 0.16, 0.40, 1.0 μg/kg; gd 15; po	Rat Holtzman	in 1.0 ug/kg grp 8% decr live births; decr pup wt gain & feed consumption to weaning in 0.40 & 1.0 μg/kg offspring; in male offspring dose-dependent decr anogenital distance at pnd 1 & 4, delayed testes descent; at 0.16 ug/kg and above decr juvenile, pubertal, postpubertal & mature seminal vesicle and ventral prostate wt and plasma testosterone and 5α-dihydrotestosterone		Mably et al. 1992a
2,3,7,8-tetrachlorodibenzo-p-dioxin	0.064, 0.16, 0.40, 1.0 μg/kg; gd 15; po	Rat Holtzman	dose-dependent incr in mount, intromission, & ejaculation latencies and decr copulatory rates in male offspring from 0.16 μg/kg and above		Mably et al. 1992b

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
2,3,7,8-tetrachlorodibenzo-p-dioxin	0.064, 0.16, 0.40, 1.0 μg/kg; gd 15; po	Rat Holtzman	in castrated male offspring treated w/ estradiol benzoate (20 μg, sc) & progesterone (1 mg, sc) dose-related incr in lordosis quotient & intensity of response to being mounted from 0.16 μg/kg and above; dose-related incr in plasma LH surge		Mably et al. 1992b
2,3,7,8-tetrachlorodibenzo-p-dioxin	0.064, 0.16, 0.40, 1.0 μg/kg; gd 15; po	Rat Holtzman	in male offspring dose-related decr epididymis & cauda epididymis wt , decr sperm production (juvenile to maturity), decr cauda epididymal sperm reserves (postpubertal to maturity) at 0.064 μg/kg grp & above; dose-related decr testis wt from juvenile to maturity	decr spermatogenesis due to impaired division and/or incr attrition of cells during conversion of leptotene spermatocytes to spermatozoa and/or by reduction in Sertoli cell no.	Mably et al. 1992c
2,3,7,8-tetrachlorodibenzo-p-dioxin	0.064, 0.16, 0.40, 1.0 μg/kg; gd 15; po	Rat Holtzman	in male offspring decr anogenital distance, delayed testis descent, decr seminal vesicle wt at 0.16 μg/kg and above; decr prostate wt at 0.064 μg/kg and above; dose-related decr epididymis, caudal epididymis wts, epididymal sperm reserves, daily sperm production, & seminiferous tubule diameter at 0.64 μg/kg and above, decr testis wt at 0.40 μg/kg and above; incr mount, intromission, & ejaculatory latencies at 0.16 μg/kg and above; incr no. of mounts & intromissions at 0.40 μg/kg & above; decr copulatory rate, incr postejaculatory interval, incr lordosis quotient, and intensity score at 0.40 μg/kg; 95% decr plasma LH pnd 32 at 1.0 μg/kg	incomplete sexual differentiation of CNS due to decr plasma testosterone concentrations before and shortly after birth	Mably et al. 1991

Table 6. Selected Studies of Androgen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	vinclozolin	10, 30, 100 mg/kg/d; pnd 22; po	Rat Long Evans	delayed preputial separation & decr epididymal wt in 30 & 100 mg/kg grps; decr ventral prostate & seminal vesicle wt, & decr sperm count in whole epididymis in 100 mg/kg grp; incr serum LH at all doses, incr T and 5α-androstanediol concentrations at 100 mg/kg; decr high to low salt AR ratio in 100 mg/kg grp for epididymis & ventral prostate, and in 30 mg/kg grp for seminal vesicles; active metabolites found in serum of animals in 30 & 100 mg/kg grps	blocks androgen-induced gene expression	Monosson et al. 1999
2	di(n-butyl)phthalate	0.5, 5, 50, 100, 500 mg/kg/d; gd 12-21; po	Rat Sprague Dawley	in male offspring of 500 mg/kg/d grp decr anogenital distance, incr cleft penis; absent of partially developed epididymis, vas deferens, seminal vesicles, & ventral prostate; decr testis, epididymis, dorsolateral & ventral prostate, seminal vesicles, and levator anibulbocavernosus muscle (pnd 110); seminiferous tubule degeneration, focal interstitial cell hyperplasia, and interstitial cell adenoma; retained areolas or nipples in 100 & 500 mg/kg/d male offspring		Mylchreest et al. 2000
3	di(n-butyl)phthalate	100, 250, 500 mg/kg/d; gd 12-21; po	Rat Sprague Dawley	in male offspring of 500 mg/kg/d grp hypospadias; cryptorchidism; agenesis of prostate ,epididymis, and vas deferens; degeneration of seminiferous epithelium; interstitial cell hyperplasia of testis; interstitial cell adenoma at 500 mg/kg/d; delayed preputial separation at 100 mg/kg/d		Mylchreest et al. 1999
4	flutamide	100 mg/kg/d; gd 12-21; po	Rat Sprague Dawley	in male offspring of 250 & 500 mg/kg/d grp retained thoracic nipples, decr anogenital distance; incr inguinal testes;		Mylchreest et al. 1999
5	linuron	0, 12.5, 25, 50 mg/kg/d; gd 12-21; po	Rat Sprague Dawley	in male offspring dose-related incr nipple retention; incr hypoplastic testes & epididymides in 12.5 mg/kg grp and above; partial agenesis of epididymides in 50 mg/kg grp	preferential impairment of testosterone-mediated, rather than DHT-mediated reproductive development	McIntyre et al. 2000

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
2,3,7,8-tetrachlorodibenzo-p-dioxin	0.064, 0.16, 0.40, 0.1 μg/kg; gd 15	Rat strain not specified	demasulinzed sexual behavior; dose-related incr in mount, intromission, & ejaculation latencies; decr copulatory rates; after castration and estradiol benzoate priming & progesterone feminized sexual behavior including dose-related incr lordosis frequency & intensity when mounted; feminization of LH secretion		Peterson et al. 1992
carbofuran	0.4 mg/kg; gd 0 - 21; po	Rat Druckrey	in male offspring decr SDH & incr LDH and γ–GT activites; decr % sperm motility, sperm count; incr % abnormal sperm; loss of spermatogenesis, degenerative changes in Sertoli cells	effects may be due to transfer of carbofuran or its metabolites through placenta; interferes w/ testes maturation	Pant et al. 1997
carbofuran	0.2, 0.4 mg/kg; pnd 0 - 21; po	Rat Druckrey	in male offspring decr SDH & incr LDH and γ–GT activites at 0.4 mg/kg; decr % sperm motility, sperm count; incr % abnormal sperm at 0.4 mg/kg; moderate tubular edema and changes in Sertoli cells at 0.4 mg/kg	effects may be due to transfer of carbofuran or its metabolites through mothers milk; interferes w/ testes maturation	Pant et al. 1997
vinclozolin	100 mg/kg; 14 d (22-23 or 35-36 days old); po	Rat Alpk:APfSD	decr. combined epididymide wt.		Ashby and Lefevre, 2000
cyproterone acetate	25 mg/kg; 14 d (22-23 days old); po	Rat Alpk:APfSD	decr. final body wt., incr liver, combined testes, combined epididymides, combined seminal & vesicles, prostate wts.		Ashby and Lefevre, 2000
cyproterone acetate	25 mg/kg; 14d (35-36 days old); po	Rat Alpk:APfSD	decr. final body wt., decr liver, combined testes, combined epididymides, combined seminal & vesicles, prostate wts.		Ashby and Lefevre, 2000
flutamide	25 mg/kg; 14d (22-23 or 35-36 days old); po	Rat Alpk:APfSD	incr. liver wt.; decr. combined epididymide combined seminal vesicle & prostate wt		Ashby and Lefevre, 2000
2,2-bis-(4-chlorophenyl)-1,1-dichloroethylene	100 or 150 mg/kg; 14 or 34d (22-23 days old); 100 or 150 mg/kg; 20d (35-36 days old); po	Rat Alpk:APfSD	incr. liver wt		Ashby and Lefevre, 2000

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
2,2-bis(4-chlorophenyl)-1,1-dichloroethylene	100 mg/kg; 14d (22-23 days old) 20 day recovery; po	Rat Alpk:APfSD	decr. final body wt		Ashby and Lefevre, 2000
dibutyl phthalate (DBP)	500 mg/kg; 14 d (22-23 days old); po	Rat Alpk:APfSD	incr liver wt; decr combined testes & combined seminal vesicle wt		Ashby and Lefevre, 2000
dibutyl phthalate (DBP)	500 mg/kg; 20 d (35-36 days old) po	Rat Alpk:APfSD	incr liver wt		Ashby and Lefevre, 2000
dibutyl phthalate (DBP)	500 mg/kg; 14 d (22-23 days old); w/ recovery; po	Rat Alpk:APfSD	decr combined testes & combined epididymide wt.		Ashby and Lefevre, 2000
dibutyl phthalate (DBP)	500 mg/kg; 34 d (22-23 days old); po	Rat Alpk:APfSD	incr liver & kidney wt; decr combined epididymides & combined seminal vesicle wt		Ashby and Lefevre, 2000
linuron	25, 125, 625 ppm; oral	Rat Rochester strain (ex-Wistar, 1923)	no reproductive malformations reported over 3 generations of up to 125 ppm levels		Hodge et al. 1968
p,p-DDE	100 mg/kg/d; gd 14-18; gavage	Rat Long Evans	showed antiandrogenic activity both *in vivo* and *in vitro*		Kelce et al. 1995
vinclozolin	100 mg/kg/d; gd 14-pnd 3; oral	Rat Sprague-Dawley	demasculinizes and feminizes the male offspring		Kelce et al. 1994
vinclozolin	200 mg/kg/d; 5 d; gavage	Rat Sprague-Dawley	induced reciprocal decline in seminal vesicle and prostate weight; inhibit expression of two specific androgen-regulated genes (TRPM-2 and C3) in the rat ventral prostate		Kelce et al. 1997
p,p'-DDE	200 mg/kg/d; 5 d; gavage	Rat Sprague-Dawley	inhibits androgen-dependent gene expression *in vivo*		Kelce et al. 1997
linuron	12.5, 25, 50, 100, 200 mg/kg; gd6-15; gavage	Rat Wistar	no significant effects reported		Khera et al. 1978
2,3,7,8-tetrachlorodibenzo-p-dioxin	121 ng; oral	Rat Wistar	0.13% retention of total dose in fetuses		van den Berg et al. 1987

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
2,3,7,8-tetrachlorodibenzo-p-dioxin	1.0 μg/kg gd 15 po	Rat Holtzman	in female pups on pnd 21 decr ovarian AHR mRNA and DNA-binding capability; incr uterus message and DNA binding: incr hypothalamic DNA-binding activity		Chaffin and Reinhold 1997
linuron	200 mg/kg/d 2 wks po	Rat CD	decr accessory sex organ wt in sexually immature & mature rats, incr serum estradiol and LH levels in mature rats	linuron produces Leydig cell tumors by an antiandrogenic mechanism where sustained hypersecretion of LH appears to be responsible for the development of Leydig cell hyperplasia & adenomas	Cook et al. 1993
flutamide	10 mg/kg/d 2 wks sc	Rat CD	decr accessory sex organ wt in sexually immature & mature rats, incr serum estradiol and LH levels in mature rats; elevated testosterone level		Cook et al. 1993
linuron	12.5, 100, 625 ppm 62-64 d (P1 males) 147-161d (F1 males) in diet	Rat CD	incr serum estradiol and LH levels in P1 and F1 males		Cook et al. 1993
di-n-butyl phthalate	0.5, 0.63, 0.75, 1.0 g/kg gd 7-15 po	Rat Wistar	complete resorption of implanted embryos in surviving dams in 1.0 g/kg grp; in 0.63 & 0.75 g/kg grps incr postimplantation loss, & decr fetal wt; incr incidence of cleft palate in fetuses of 0.75 g/kg grp		Ema et al. 1993
di-n-butyl phthalate	0.75, 1.0, 1.5 g/kg gd 7-9 po	Rat Wistar	100% postimplantation loss at 1.5 g/kg; incr postimplanation loss at 0.75 & 1.0 g/kg; incr skeletal malformations at 0.75 & 1.0 g/kg		Ema et al. 1994
di-n-butyl phthalate	0.75, 1.0, 1.5 g/kg gd 10-12 po	Rat Wistar	100% postimplantation loss at 1.5 g/kg; incr postimplanation loss at 0.75 & 1.0 g/kg		Ema et al. 1994
di-n-butyl phthalate	0.75, 1.0, 1.5 g/kg gd 13-15 po	Rat Wistar	100% postimplantation loss at 1.5 g/kg; incr postimplanation loss at 0.75 & 1.0 g/kg; dose dependent incr external & skeletal malformations		Ema et al. 1994
dimethyl phthalate	1.4 g/kg/d 4 d po	Rat Sprague-Dawley	no testicular effects; no effects on urinary zinc excretion or testicular zinc content		Foster et al. 1980

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
diethyl phthalate	1.6 g/kg/d 4 d po	Rat Sprague-Dawley	no testicular effects; no effects on urinary zinc excretion or testicular zinc content		Foster et al. 1980
dipropyl phthalate	1.8 g/kg/d 4 d po	Rat Sprague-Dawley	no testicular effects; no effects on urinary zinc excretion or testicular zinc content		Foster et al. 1980
di-n-butyl phthalate	2.0 g/kg/d 4 d po	Rat Sprague-Dawley	no testicular effects; no effects on urinary zinc excretion or testicular zinc content		Foster et al. 1980
di-n-pentyl phthalate	2.1 g/kg/d 4 d po	Rat Sprague-Dawley	testicular atrophy; incr urinary zinc excretion; decr testicular zinc content		Foster et al. 1980
di-n-hexyl phthalate	2.4 g/kg/d 4 d po	Rat Sprague-Dawley	testicular atrophy; incr urinary zinc excretion; decr testicular zinc content		Foster et al. 1980
di-n-heptyl phthalate	2.6 g/kg/d 4 d po	Rat Sprague-Dawley	no testicular effects; no effects on urinary zinc excretion or testicular zinc content		Foster et al. 1980
di-n-octyl phthalate	2.8 g/kg/d 4 d po	Rat Sprague-Dawley	no testicular effects; no effects on urinary zinc excretion or testicular zinc content		Foster et al. 1980
16β-bromo-3β,17α-dihydroxy-5α-pregnane-11,20-dione	50 mg/kg 3 x/ 2 d sc	Rat Sprague-Dawley	inhibition of 17α-hydroxylase & C17-20 lyase;		Goldman et al. 1976
17β-ureido–1,4-androstadien-3-one)	50 mg/kg 3 x / 2 d sc	Rat Sprague-Dawley	inhibition of 17α-hydroxylase & C17-20 lyase		Goldman et al. 1976
16β-bromo-3β,17α-dihydroxy-5α-pregnane-11,20-dione	30 mg/kg 1 x/d gd 13-15 sc	Rat Sprague-Dawley	hypospadias in male offspring		Goldman et al. 1976
17β-ureido–1,4-androstadien-3-one)	10-30 mg/kg 1 x/d gd 13-20 sc	Rat Sprague-Dawley	hypospadias in male ofspring		Goldman et al. 1976
17β-ureido–1,4-androstadien-3-one)	600 υg pnd 1-9 sc	Rat Sprague-Dawley	decr prostate & seminal vesicle wt in adulthood		Goldman et al. 1976

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
2,3,7,8-tetrachlorodibenzo-p-dioxin	1 µg/kg gd 8 or 15 po	Rat Long Evans	delayed puberty, complete to partial clefting of the phallus, permanent vaginal thread in gd 15 grp female offspring; partial cleft phallus & vaginal thread in gd 8 grp females; decr ovarian wts in gd 8 & 15 offspring; mating difficulties & vaginal bleeding in gd 15 females; incr constant estrus, cystic endometiral hyperplasia, & quicker decr fertility rate in gd 8 grp		Gray and Ostby 1995
2,3,7,8-tetrachlorodibenzo-p-dioxin	1 µg/kg gd 15 po	Rat Holtzman	clefting of phallus in all female offspring; vaginal thread; decr ovarian wt		Gray and Ostby 1995
2,3,7,8-tetrachlorodibenzo-p-dioxin	1 µg/kg gd 8 po	Rat Long Evans	delayed preputial separation; decr ejaculated sperm count; decr epididymal sperm storage; decr accessory gland wt; decr anogenital distance; altered male sex behavior	alterations not likely due to androgen status since testosterone and androgen receptor levels were normal	Gray et al. 1995
2,3,7,8-tetrachlorodibenzo-p-dioxin	2 µg/kg gd 11 po	Hamster Syrian	delayed preputial separation; decr ejaculated sperm count; decr epididymal sperm storage	alterations not likely due to androgen status since testosterone and androgen receptor levels were normal	Gray et al. 1995
2,3,7,8-tetrachlorodibenzo-p-dioxin	0.05, 0.20, 0.80 µg/kg gd 15 po	Rat Long Evans	delayed puberty in 0.2 & 0.8 µg/kg grps; transient decr ventral prostate & seminal vesicle wt in all trt grps; decr epididymal sperm reserves & glans penis size in all trt grps; 45%, 25%, 25% decr ejaculated sperm in the 0.8, 0.2, and 0.05 µg/kg grps.		Gray et al. 1997a
2,3,7,8-tetrachlorodibenzo-p-dioxin	0.05, 0.20, 0.80 µg/kg gd 15	Rat Long Evans	delayed vaginal opening in 0.80 µg/kg grp; vaginal thread, cleft phallus, incr urethral slit, incr distance from urethral opening to phallus tip, decr distance from urethral opening to vaginal orifice in 0.20 & 0.80 µg/kg grps; incr time to pregnancy and histopathological alterations of reproductive tract in 0.80 µg/kg grp;		Gray et al. 1997b
2,3,7,8-tetrachlorodibenzo-p-dioxin	1.0 µg/kg gd 15	Rat Long Evans	incr cleft phallus & vaginal thread; decr ovarian & pituitary wt	effects due to in utero exposure not lactational exposure	Gray et al. 1997b

Table 6. Selected Studies of Androgen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	benzyl butyl phthalate	0.75 g/kg gd 14- pnd 3 po	Rat Sprague-Dawley	decr anogenital distance & testis wt, nipple/aereola retention, & reproductive malformations in male offspring		Gray et al. 2000
2	diethylhexyl phthalate	0.75 g/kg gd 14- pnd 3 po	Rat Sprague-Dawley	decr anogenital distance & testis wt, nipple/aereola retention, & reproductive malformations in male offspring		Gray et al. 2000
3	diocty tere-phthalate	0.75 g/kg gd 14- pnd 3 po	Rat Sprague-Dawley	no significant effects		Gray et al. 2000
4	diethyl phthalate	0.75 g/kg gd 14- pnd 3 po	Rat Sprague-Dawley	no significant effects		Gray et al. 2000
5	dimethyl phthalate	0.75 g/kg gd 14- pnd 3 po	Rat Sprague-Dawley	no significant effects		Gray et al. 2000
6	diisononyl phthalate	0.75 g/kg gd 14- pnd 3 po	Rat Sprague-Dawley	nipple/aereola retention, & reproductive malformations in male offspring		Gray et al. 2000
7	vinclozolin	200 mg/kg/d 4d po	Rat Sprague-Dawley	decr seminal vesicle & prostate wt; reduced immunohistochemical staining of AR in epididymal nuclei; induced TRPM-2 mRNA & repressed C3 mRNA; effects reduced in castrated rats	acts as an antinadrogen by altering the expression of androgen-dependent genes	Kelce et al. 1997
8	p,p'-DDE	200 mg/kg/d 4d po	Rat Sprague-Dawley	decr seminal vesicle & prostate wt; reduced immunohistochemical staining of AR in epididymal nuclei; induced TRPM-2 mRNA & repressed C3 mRNA; effects reduced in castrated rats	acts as an antinadrogen by altering the expression of androgen-dependent genes	Kelce et al. 1997
9						
10						
11	linuron	100 mg/kg/d 7 d po	Rat Sprague-Dawley	in Hershberger Assay decr seminal vesicle, ventral prostate gland, levator ani plus bulbocavernosus muscle wts in castrated males		Lambright et al. 2000
12	linuron	100 mg/kg/d 7 d po	Rat Sprague-Dawley	decr ventral prostate ,epiddymal and levator ani plus blubocavernousus wts		Lambright et al. 2000

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
linuron	100 mg/kg/d 4 d po	Rat Sprague-Dawley	altered expression of androgen-regulated ventral prostate genes (incr TRPM2 & decr C3 mRNA)		Lambright et al. 2000
linuron	100 mg/kg/d gd 14-18 po	Rat Sprague-Dawley	agenesis and/or atrophy of testes & epididymides; testicular & epididymal lesions; no active seminiferous tubule spermatogenesis in 62%		Lambright et al. 2000
di-n-butyl phthalate	500 mg/kg/d gd 14 -pnd 3 po	Rat Sprague-Dawley	agenesis and/or atrophy of testes & epididymides; testicular & epididymal lesions; no active seminiferous tubule spermatogenesis in 50%		Lambright et al. 2000
diethylhexyl phthalate	750 mg/kg/d gd 14 - pnd 3	Rat Sprague-Dawley	decr testosterone production, decr testicular & whole-body testosterone in fetal & neonatal male rats from gd 17-pnd 2; 36% decr anogenital distance pnd 2 in male offspring; decr testis wt by gd 20; enhanced 3β-HSD staining & incr multifocal areas of Leydig cell hyperplasia and mutinucleated gonocytes in testes at gd 20 & pnd 3	DEHP disrupts male rat sexual differentiation by reducing testosterone to female levels in the fetal male rat during a critical stage of reproductive tract development	Parks et al. 2000
2,3,7,8-tetrachlorodibenzo-p-dioxin	1.0 μg/kg gd 15 po	Rat Holtzman	incr cyctochrome P45001A1 mRNA & protein in whole prostates between pnd 7 & 21; transient decr in androgen-regulated prostatic mRNA at pnd 21	mRNA effects probably not the result of direct action of the activated AhR on these genes but reflective of a TCDD-induced delay in prostate development	Roman and Peterson, 1998
2,3,7,8-tetrachlorodibenzo-p-dioxin	25 μg/kg approx. pnd 65-66 po	Rat Holtzman	CYP1A1 mRNA induction		Roman and Peterson, 1998
2,3,7,8-tetrachlorodibenzo-p-dioxin	2 μg gd 11.5 po	Hamster Syrian	delayed VO & altered estrous cycles in F1 females; 20% infertility in treated F1 females & 38% mortality of pregnant F1 females near term; decr implants & live pups in treated females; external urogenital malformations including cleft phallus; decr survival of F2 treated offspring		Wolf et al. 1999
vinclozolin	400 mg/kg/d gd 12-13 po	Rat Long Evans	no significant effects		Wolf et al. 2000

Table 6. Selected Studies of Androgen Modulators (continued)

	Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
1	vinclozolin	400 mg/kg/d gd 14-15 po	Rat Long Evans	decr anogenital distance; presence of areolas, nipples, & malformation of the phallus; reduced levator anti/bulbocavernosus wt		Wolf et al. 2000
2	vinclozolin	400 mg/kg/d gd 16-17 po	Rat Long Evans	decr anogenital distance; presence of areolas, nipples, & malformation of the phallus; reduced levator anti/bulbocavernosus wt		Wolf et al. 2000
3	vinclozolin	400 mg/kg/d gd 18-19 po	Rat Long Evans	decr anogenital distance; presence of areolas, nipples, & malformation of the phallus; reduced levator anti/bulbocavernosus wt; decr ventral prostate wt.		Wolf et al. 2000
4	flutamide	40 mg/kg/d gd 14-18 po	Rat Sprague-Dawley	decr anogenital distance & retained nipples in male offspring; decr testis, epididymis, ventral prostate & seminal vesicle wt		You et al. 1998
5	flutamide	40 mg/kg/d gd 14-18 po	Rat Long Evans	decr anogenital distance & retained nipples in male offspring; decr testis, epididymis, & ventral prostate wt		You et al. 1998
6	p,p'-DDE	10, 100 mg/kg/d gd 14-18 po	Rat Sprague-Dawley	incr nipple retention in male offspring of both trt grps; altered androgen receptor expression in 100 mg/kg grp male offspring;		You et al. 1998
7	p,p'-DDE	10, 100 mg/kg/d gd 14-18 po	Rat Long Evans	decr anogenital distance & incr nipple retention in 100 mg/kg grp male offspring; altered androgen receptor expression in 100 mg/kg grp male offspring		You et al. 1998
8	p,p'-DDE	10, 100 mg/kg/d gd 14-18 po	Rat Sprague Dawley	fetal DDE 3-fold higher than placental concentrations; in 100 mg/kg grp liver DDE concentration detectable on pnd 10 w/ lactational exposure grp concentrations 50x higher than in utero only	mobilization of DDE from its storage sites is suspected to occur through its association w/ mobilized fatty acids & lipoproteins	You et al 1999
9	vinclozolin	400 mg/kg/d gd 20-21 po	Rat Long Evans	no significant effects		Wolf et al. 2000

Table 6. Selected Studies of Androgen Modulators (continued)

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
vinclozolin	200, 400 mg/kg/d gd 14-19 po	Rat Long Evans	in males from both dose grps decr anogenital distance; presence of areolas, nipples, genital malformations, decr ventral prostate and seminal vesicle wt, vaginal pouch; ectopic/undescended testes in 400 mg/kg/d grp		Wolf et al. 2000

γ-GT = γ-glutamyl dehydrogenase
AR = androgen receptor
CNS = central nervous system
d = days
decr = decreased
FSH = follicle stimulating hormone
gd = gestational day
grp = grp
GSH = glutathione
hCG = human chorionic gonadotropic
inc = increased
ld = lactation day
LDH = lactate dehydrogenase
LH = luteinizing hormone
pc = post coitum
pd = postpartum day
pnd = postnatal day
po = gavage
PPS = preputial separation
sc = subcutaneous
SDH = sorbitol dehydrogenase
TSH = thyroid stimulating hormone
VO = vaginal opening
w/o = without
w/ = with
wks = weeks
wt = weight

Table 7. Selected Studies of Other Endocrine Active Compound Modulators

Treatment	Dose (duration and route)	Species/Strain	Effect	Putative Mechanism	Reference
6-propylthiouracil (PTU) (potent thyroid modulator)	240 mg/kg/day; pnd 21-51; po	Rat CD	delayed PPS and growth; decreased testis and epididymis weights, decreased relative thyroid weight, decreased serum T4, DHT, and testosterone levels, increased TSH levels and relative thyroid weight, and altered thyroid histology indicative of increased TSH secretion	Inhibition of thyroid hormone synthesis	Marty et al., 2001
phenobarbital (PB) (weak thyroid modulator)	50 or 100 mg/kg/day; pnd 21-51; po	Rat CD	decreased absolute testis, epididymis, prostate and seminal vesicle weights at high dose; increased relative thyroid weight, decreasd T4.	Accelerated throxine metabolism and clearance through induction of hepatic microsomal enzymes	Marty et al., 2001
haloperidol (HALO) (dopamine antagonist)	2 or 4 mg/kg/day; pnd 21-51; po	Rat CD	Increased relative thyroid weight and decreased T4		Marty et al., 2001
bromocryptine (BRC) (dopamine agonist)	10 or 50 mg/kg/day; pnd 21=51; po	Rat CD	PPS and growth delayed at high dose; decreased absolute prostate and seminal vesicle weight;		Marty et al., 2001
bromocriptine w/ young & adult pituitary grafts (dopamine agonist)	4 mg/kg; 2x/daily from day 21; sc	Rat Wistar	early PPS & incr prolactin w/ adult & young graft; bromocriptine decr prolactin in both grps & blocked early puberty for adult graft animals; bromocriptine reduced plasma testosterone in adult graft grp	bromocriptine inability to block early puberty in young graft animals suggests effect is mediated by a factor other than prolactin	Aguilar et al. 1988

γ-GT = γ-glutamyl dehydrogenase
AR = androgen receptor
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FSH = follicle stimulating hormone
gd = gestational day
grp = grp
GSH = glutathione
hCG = human chorionic gonadotropic
inc = increased
ld = lactation day
LDH = lactate dehydrogenase
LH = luteinizing hormone
pc = post coitum
pd = postpartum day
pnd = postnatal day
po = gavage
PPS = preputial separation
sc = subcutaneous
SDH = sorbitol dehydrogenase
TSH = thyroid stimulating hormone
VO = vaginal opening
w/o = without
w/ = with
wks = weeks
wt = weight

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